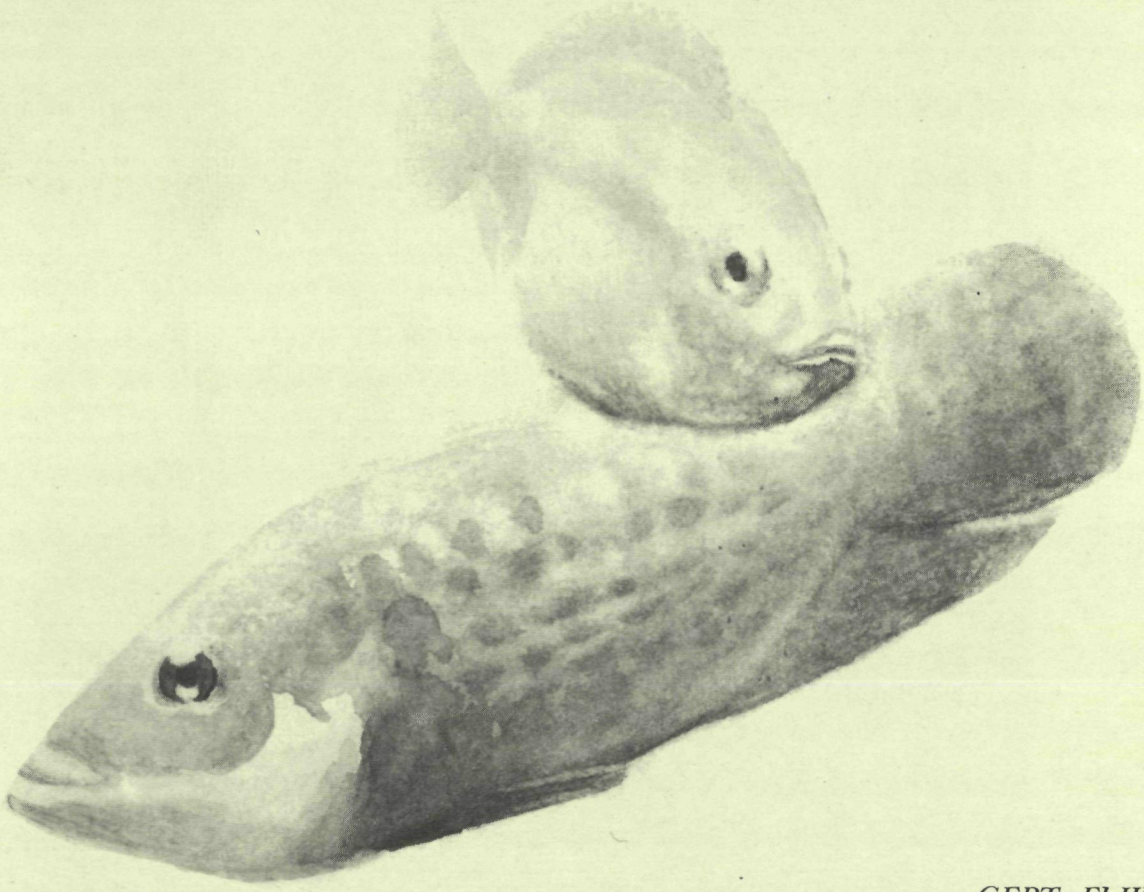
CALCIUM BALANCE IN FRESHWATER TELEOST FISH:

Ca^{2+}-UPTAKE MECHANISMS AND INTERNAL DISTRIBUTION OF CALCIUM.



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CALCIUM BALANCE IN FRESHWATER TELEOST FISH: Ca^{2+}-UPTAKE MECHANISMS AND INTERNAL DISTRIBUTION OF CALCIUM

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CALCIUM BALANCE IN FRESHWATER TELEOST FISH: Ca^{2+}-UPTAKE MECHANISMS AND INTERNAL DISTRIBUTION OF CALCIUM

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CONTENTS

PART I

MECHANISMS FOR ACTIVE Ca^{2+}-TRANSPORT IN THE GILLS OF FRESHWATER TELEOST FISH

ADP : adenosinediphosphate

AMP : adenosinemonophosphate

ATP : adenosinetriphosphate

ATPase: adenosinetriphosphate phosphohydrolase

EDTA : (ethylenedinitrilo)tetraacetic acid

EGTA : (ethylene-bis(oxyethylenenitrilo))tetraacetic acid

Hepes : N-2-hydroxyethylpiperazine-N´-2-ethanesulphonic acid

Me^{2+} : Ca^{2+} or Mg^{2+}

MS222 : tricaine methanosulphonate

p-NPP : para-nitrophenylphosphate

NPPase: para-nitrophenylphosphate phosphohydrolase

NTA : nitrilotriacetic acid

PMSF : phenylmethylsulphonylfluoride

R24571: 1-[bis(p-chlorophenyl)-methyl]-3-2(2,4-dichloro-β-(2,4-dichlorobenzyloxy)-phenethyl)imidazoliumchloride

TCA : trichloroacetic acid

Tris : tris(hydroxymethyl)aminomethane

CHAPTER I

GENERAL INTRODUCTION

Importance of calcium for freshwater fish

Teleost fish inhabit every conceivable marine niche and in fresh waters they actually represent the total population of bony fishes. Their success in evolution, perhaps best indicated by the fact that they estimate some 30.000 recent species (versus e.g. 4000 recent mammalian species) is ascribed to their efficient anatomy, exceptional fertility and remarkable physiological adaptability (Storer *et al.*, 1979; Romer, 1962). The research presented in this thesis fits in with the adaptability of fish. With respect to their capacity for adaptation fish are often subdivided into stenohaline species (e.g. herring and carp) and euryhaline species (e.g. salmon and eel), which terms refer to a narrow and a broad salt tolerance, respectively.

A euryhaline fish, whether in fresh water or in sea water, shows osmotic homeostasis. Although this homeostasis may not be as strict as that observed in mammals, plasma ion composition and plasma osmolarity in most fish resemble those in mammals (Simkiss, 1974). Freshwater fish, being submerged in a hypo-osmotic environment, perform precise control of integumental permeability and produce large amounts of hypotonic urine to compensate for water influx; they take up ions from the water to balance outward diffusion of ions from the body.

For a long time, research on osmo-ionic balance in fish has focussed solely on the fish's handling of sodium, chloride and water. The crucial function of calcium, in particular for the physiology of freshwater fish, has long been underestimated. Ever since the early seventies, Wendelaar Bonga and co-workers in Nijmegen in conjunction with others (J.C. Fenwick, Canada; T. Hirano, Japan; N. Mayer-Gostan and E. Lopez, France; P.K.T. Pang, USA) have directed attention to calcium metabolism and its endocrine regulation in fish. In fish, similarly as in all vertebrates, levels of ionized calcium in the extracellular fluids are maintained within narrow limits (around 1.5 mM Ca^{2+}) for the appropriate functioning of a variety of physiological processes, such as muscle contraction, nerve-impulse conduction, hormone secretion, activation of enzymes, blood coagulation, reproduction and skeletal calcium accretion.

For fish evidence is accruing that calcium homeostasis is of pivotal importance to successfully maintain hydromineral balance. It has been recognized

that even a slight reduction of the calcium concentration in the extracellular fluids increases the permeability of the body surface to water and ions. Thus, for a fish to live and grow in fresh waters, which are generally hypocalcic to the body fluids, a permanent need exists to establish a positive calcium balance. Survival in fresh water is, therefore, only possible for fish that posses efficient mechanisms for the uptake of calcium from the environment. This requisite does not exist for seawater fish, since the calcium concentration in sea water (approximately 10 mM Ca^{2+}) is substantially higher than that in the extracellular body fluids. It seems not too imprudent to state that the invasion of fresh water by fish in the course of vertebrate evolution was only possible after the development of mechanisms for the uptake of calcium from the environment.

Location of calcium uptake mechanisms

Freshwater fish depend on their gills and gut for the uptake of calcium (Berg, 1968). Gills are of major importance for the uptake of Ca^{2+}: when fish are fed a calcium-deficient diet, growth and calcium accumulation in the body are not hampered, provided the ambient Ca^{2+}-levels are not too low (Rodgers, 1984). Moreover, freshwater fish drink very little and therefore, the gills remain as the major site for the uptake of Ca^{2+} (Fig. 1).

The gills of fish are a multifunctional organ, involved in gas- and ion-exchange between the fish and the water. The branchial epithelium consists of a thin layer of cells that separates the blood from the ambient water and forms a minimal diffusion barrier for oxygen and carbon dioxide. Cytologically, the most conspicuous cells of the branchial epithelium are the so-called ionocytes or chloride cells, which names refer to their role in ion transport. The cytoplasm of these cells contains an extensive tubular system associated with the basolateral plasma membranes and in contact with numerous mitochondria (Karnaky, 1980). This structure reminds of the well-known basal labyrinth in cells of other ion-transporting epithelia such as the mammalian nephron or the avian nasal gland. Ultracytochemically and biochemically, it has been shown that the bulk of Na^+/K^+-ATPase, the enzyme that provides for the energy for active Na^+-extrusion from the cell, is concentrated in these chloride cells (Hootman & Philpott, 1978, 1979). On the basis of experiments with freshwater trout it has been claimed by Payan *et al.* (1981) that also Ca^{2+}-influx through the gills is mediated exclusively by the chloride cells. In contrast to land-living vertebrates that take up calcium intermittently by feeding, fish have continuous access to Ca^{2+} in the ambient water. Therefore, water forms a virtually inexhaustible calcium source, however low the Ca^{2+}-con-

centrations are, and provided that the calcium uptake mechanisms are adequate.

Transfer of Ca^{2+} from the water to the blood

Little is known about the mechanisms involved in active Ca^{2+}-transport in fish gills. A "Ca^{2+}-ATPase" activity has been demonstrated in branchial epithelium of a variety of fish species. Off-handedly, this enzyme activity was presumed to form the basis for active transport of Ca^{2+} through the gills. Its kinetic parameters, however, make an involvement in active Ca^{2+}-transport unlikely. The permeability of the gills to water and ions is low and, in fact, the branchial epithelium belongs to the most impermeable epithelia known in the animal kingdom. In such a tight epithelium as that of the gills, Ca^{2+} may be expected to move from the water to the blood through the cells of the epithelium, and not via paracellular routes.

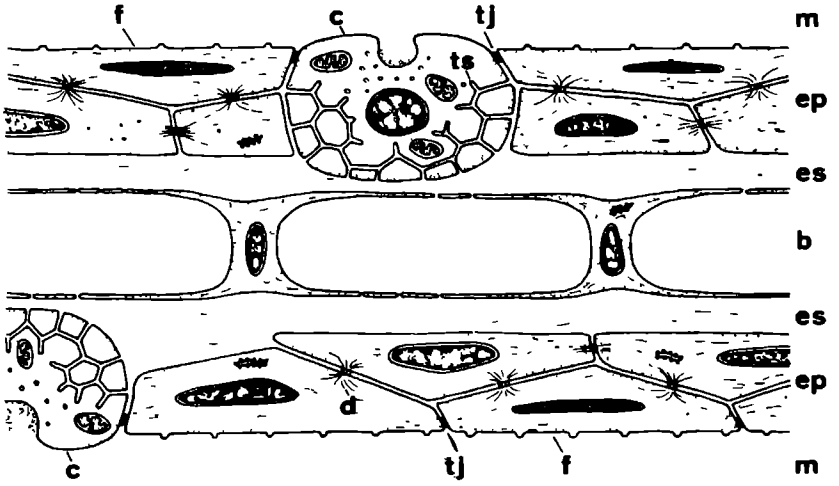

Figure 1. Cross-section of secondary gill filament of tilapia; m, mucus layer; ep, skin epithelium, with filament-containing cells (f) and chloride cells (c); the cells are connected by desmosomes (d), and, at the outer surface, by tight junctions (tj); the chloride cells contain a tubular system (ts) of membranes which is continuous with the basolateral cell membranes; es, extracellular space; b, branchial blood vessels.

Since intracellular Ca^{2+}-concentrations are generally very low (< 1 μM Ca^{2+}), Ca^{2+}-ions from the water are likely to permeate the cell down an electrochemical gradient. As a consequence, the subsequent step from the cytosol to the blood is against a steep gradient and requires active transport energized by the hydrolysis of ATP. The occurrence of transport Ca^{2+}-ATPases, such as the well-established Ca^{2+}-ATPase of erythrocyte membranes, has been demonstrated lately in the basolateral membranes of mammalian Ca^{2+}-transporting epithelia of the intestine and kidney. The presence of such transport Ca^{2+}-ATPases and their role in transepithelial Ca^{2+}-transport in the lower vertebrates needs to be established.

Hormonal control of calcium metabolism

In mammals, the minute-to-minute homeostasis of the body fluids is effected by parathormone (PTH, a hypercalcemic hormone) and calcitonin (a hypocalcemic hormone). Primary targets for these hormones are bone and kidneys (Irving, 1973). Absorption of calcium from ingested food is achieved via 1α,25-dihydroxyvitamin D_3-dependent mechanisms in the intestine (DeLuca & Schnoes, 1976). PTH, however, is the governing hypercalcemic hormone in calcium homeostasis, since it controls the synthesis of 1α,25-dihydroxyvitamin D_3 which, in turn, may facilitate PTH-actions on the bone and other target tissues (DeLuca, 1971).

The regulation of calcium metabolism in fish differs essentially from that in higher vertebrates. Fish lack parathyroid glands. Unique for fish are the so-called Stannius corpuscles, endocrine glands associated with the kidneys. Immunologically, Stannius-products exhibit cross-reactivity with antibodies raised against mammalian PTH (Milet *et al.*, 1980), which suggests structural resemblance between the putative Stannius hormone and PTH. However, the function of the Stannius-products is clearly different, for they are hypocalcemic factors.

In freshwater fish prolactin from the rostral pars distalis of the pituitary gland is recognized as a hypercalcemic hormone (Pang *et al.*, 1979; Wendelaar Bonga & Flik, 1982). Although the hypercalcemic nature of prolactin in fish seems well-established, the mechanism of action of prolactin is, in fact, completely unknown. Prolactin could stimulate mechanisms for the uptake of Ca^{2+} from the water, but also, it could stimulate resorption of Ca^{2+} from the bone to raise plasma calcium levels.

Research objectives

The aims of the studies presented in this thesis are:

- To demonstrate the presence of Ca^{2+}-uptake mechanisms in fish gills.
- To determine the rate of Ca^{2+}-uptake from the water in intact fish, in an attempt to assess the importance of the calcium concentrations of the water (and thus the relative importance of the Ca^{2+}-uptake mechanisms of the gills) for the maintenance of Ca-balance.
- To verify, whether the role of prolactin as a hypercalcemic hormone is based on stimulation of the uptake rate of Ca^{2+} from the water via regulation of branchial Ca^{2+}-uptake mechanisms.

The first part of this thesis presents biochemical studies on Ca^{2+}-dependent phosphatases in plasma membranes of branchial epithelium of the American eel, *Anguilla rostrata*, and the African tilapia, *Oreochromis mossambicus**. For characterization studies large eels were used, to obtain sufficient amounts of plasma membranes. Chapter II describes a method to isolate plasma membranes from branchial epithelium and deals with the characterization of Ca^{2+}-*activated* ATPase activities therein. These last enzymic activities, till then equated with Ca^{2+}-transport ATPase activity, were shown to represent a heterogeneous pool of non-specific phosphatases. Chapter III demonstrates the presence of Ca^{2+}-*stimulated* ATPase activities in eel gill plasma membranes. An enzymic activity with characteristics of established (mammalian) Ca^{2+}-transport ATPases could be distinguished from non-specific, Ca^{2+}-stimulated phosphatase activity. Among the characteristics of this high-affinity Ca^{2+}-ATPase are: high affinity for Ca^{2+}, ATP-preference and calmodulin-dependency. In Chapter IV, evidence is given that infusing freshwater eels with ovine prolactin leads to hypercalcemia, and that this hypercalcemia is accompanied by induction of the high-affinity Ca^{2+}-ATPase in the plasma membranes of the gills. This observation indicates that stimulation of branchial Ca^{2+}-transport is the consequence of prolactin action. Chapter V reports the presence of high-affinity Ca^{2+}-ATPase activity in tilapia gills. Moreover, direct evidence is given for ATP-dependent Ca^{2+}-transport in plasma membranes of these gills. The magnitude of this active Ca^{2+}-transporting process is consistent with a direct involvement of high-affinity Ca^{2+}-ATPase in transepithelial Ca^{2+}-transport *in vivo*.

The second part of this thesis concerns Ca^{2+}-exchange processes between tilapia and water and presents analyses of internal calcium-pools of the fish.

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* This species has been renamed twice recently: *Tilapia mossambica* was changed first to *Sarotherodon mossambicus* and subsequently to *Oreochromis mossambicus* (Trewavas, 1981). In our reports we mostly refer to this species as tilapia.

Chapter VI is a characterization of bidirectional Ca^{2+}-fluxes between fish and water, measured in intact fish, the emphasis being on the role of the gills. It is demonstrated that in actively feeding, growing tilapia sufficient Ca^{2+} is taken up from the water via the gills to permit the growth rates observed in these fish. Chapter VII deals with the effects of low levels of Ca^{2+} in fresh water on Ca^{2+}-fluxes and on internal calcium stores in tilapia. Fish acclimated to fresh water with a reduced calcium level, showed a clear adaptive response to this altered environment, viz. an enhancement of branchial uptake rates of Ca^{2+}. Chapter VIII is a report on the effects of prolactin on calcium exchange with the water and on internal calcium stores. The results obtained - stimulation of branchial uptake rates of Ca^{2+}, accompanied by hypercalcemia and enhanced Ca-deposition in the bone - corroborate the conclusion drawn in Chapter IV, that the stimulation by prolactin of branchial high-affinity Ca^{2+}-ATPase activity underlies the hypercalcemia observed after prolactin treatment. Chapter IX is a general discussion.

REFERENCES

BERG, A. (1968) Studies on the metabolism of calcium and strontium in freshwater fish. I- Relative contribution of direct and intestinal absorption. Mem.Ist. Ital.Idrobiol. 23: 161-196.

DeLUCA, H.F. (1971) The role of vitamin D and its relationship to parathyroid hormone and calcitonin. Recent Prog.Horm.Res. 27: 479-510.

DeLUCA, H.F. & SCHNOES, H.K. (1976) Metabolism and mechanisms of action of vitamin D. Ann.Rev.Biochem. 45: 631-666.

HOOTMAN, S.R. & PHILPOTT, C.W. (1978) Rapid isolation of chloride cells from pinfish gill. Anat.Rec. 190(3): 687-702.

HOOTMAN, S.R. & PHILPOTT, C.W. (1979) Ultracytochemical localization of Na^+/K^+-activated ATPase in chloride cells from the gills of a euryhaline teleost. Anat.Rec. 193: 99-130.

IRVING, J.T. (1973) Calcium and phosphorus metabolism. Academic Press. New York.

KARNAKY, K.J. Jr. (1980) Ion-secreting epithelia: chloride cells in the head region of *Fundulus heteroclitus*. Am.J.Physiol. 238: R185-R198.

MILET, C., HILLYARD, C.J., MARTELLY, E., GIRGIS, S., MacINTYRE, I. & LOPEZ, E. (1980) Similitudes structurales entre l'hormone hypocalcémiante des corpuscules de Stannius (PCS) de l'anguille *(Anguilla anguilla* L.) et l'hormone parathyroidienne mammalienne. C.R.Acad.Sci.P. 291: 977-980.

PANG, P.K.T., SCHREIBMAN , M.P., BALBONTIN, F. & PANG, R.K. (1979) Prolactin and pituitary control of calcium regulation in the killifish, *Fundulus heteroclitus*. Gen.Comp.Endocrinol. 36: 306-316.

PAYAN, P., MAYER-GOSTAN, N. & PANG, P.K.T. (1981) Site of calcium uptake in the freshwater trout gill. J.exp.Zool. 216: 345-347.

RODGERS, D.W. (1984) Effects of ambient pH and calcium concentration on growth and calcium dynamics of brook trout, *Salvelinus fontinalis*. Can.J.Fish & Aqua. Sci. Submitted.

ROMER, A.S. (1962) The vertebrate body. Saunders Company, Philadelphia/London.

SAMMON, P.J., STACEY, R.E. & BRONNER, F. (1970) Role of parathyroid hormone in calcium homeostasis and metabolism. Am.J.Physiol. 218: 479-485.

SIMKISS, K. (1974) Calcium metabolism of fish in relation to ageing. Proc.Int.Symp. Ageing in Fish (Bagenal ed.) Unwin Bros. Old Woking.
STORER, T.I., USINGER, R.L., STEBBINS, R.C. & NYBAKKEN, J.W. (1979) General Zoology. McGraw-Hill Book Company (Vastyan, J.E., Wagner, J. & Wagley, S. eds.) New York.
TREWAVAS, E. (1981) Nomenclature of the tilapias of Southern Africa. J.Limnol. Soc.Sth.Afr. 7: 42.
WENDELAAR BONGA, S.E. & FLIK, G. (1982) Prolactin and calcium metabolism in a teleost fish. In: Comparative Endocrinology of Calcium regulation (Oguro, C. & Pang, P.K.T. eds.) Tokyo, Japan Scientific Societies Press: 21-26.

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Ca^{2+}-DEPENDENT PHOSPHATASE AND Ca^{2+}-DEPENDENT ATPase ACTIVITIES IN PLASMA MEMBRANES OF EEL GILL EPITHELIUM. I. IDENTIFICATION OF Ca^{2+}-ACTIVATED ATPase ACTIVITIES WITH NON-SPECIFIC PHOSPHATASE ACTIVITIES.

ABSTRACT

1. The characteristics of Ca^{2+}-activated ATPase activities previously often postulated as components for the calcium transporting system in fish gills do not fulfil the requirements of a transport Ca^{2+}-ATPase.
2. The chelation of Ca^{2+}- or Mg^{2+}-ions is a prerequisite for the adenosinephosphate esters to serve as substrate for gill plasma membrane phosphatases.
3. Ca^{2+}-activated ATP hydrolysis results from the activity of a heterogeneous pool of phosphatases located in the plasma membranes of the branchial epithelium, as is concluded from substrate specificity tests and the effects of various inhibitors on these hydrolytic activities.
4. In the present study only non-specific phosphatases could be shown.

INTRODUCTION

Teleost fish, whether in fresh water (FW) or sea water (SW), possess efficient mechanisms to maintain blood calcium levels (Wendelaar Bonga & Van der Meij, 1980). In FW, under normal conditions but especially during periods of starvation, this control probably involves the active transfer of calcium from the ambient medium into the extracellular fluids. Indeed, it is well known that fish can extract calcium from their external environment and there is ample evidence to suggest that the major site for this uptake is associated with the branchial apparatus (Mashiko & Jozuka, 1964a; Berg, 1968). The mechanism of this transfer, however, has not been adequately studied. One of us (Fenwick, 1976, 1979), among others (Ma *et al.*, 1974; Moon, 1978; Ho & Chan, 1980; Doneen, 1981) described the presence in the gills of a Ca^{2+}-activated adenosinetriphosphatase (Ca^{2+}-ATPase) which, in analogy to the Na^+-transporting ouabain-sensitive Na^+/K^+-ATPase, was presumed to be the enzymic basis for the active transport of calcium through the

gills. This presumption was supported by various observations. Most significantly, the influx of $^{45}Ca^{2+}$ in perfused, isolated, American eel gills correlated positively with Ca^{2+}-ATPase activity (Fenwick, 1976; So & Fenwick, 1977). Additionally, the activity of this enzyme was reported to be influenced directly by hypocalcin (Ma & Copp, 1978), a hormone which can markedly influence calcium metabolism in teleosts. Further, the level of enzymic activity varied with ambient calcium concentration (Fenwick, 1978).

However, there are serious difficulties with this thesis. If active transport of calcium occurs in the gills it most likely occurs at the basal or lateral membranes where the calcium moves from an intracellular calcium concentration of about 10^{-7} M into a body fluid calcium concentration of about $3x\ 10^{-3}$ M. Active transport would not be required to move the calcium from the ambient medium (10^{-4} M) into the intracellular compartment (10^{-7} M). However, the typical apparent affinity ($K_{0.5}$) of the gill Ca^{2+}-ATPase has been reported as about 0.45 mM Ca^{2+}, a value which is not commensurate with effective functioning at the basal or lateral membrane sites. Secondly, the reported pH-optima for these activities were about 8.0 (Ma *et al.*, 1974; Fenwick, 1976) and were therefore within the favorable range for alkaline phosphatase activities. Thirdly, the Ca^{2+}-stimulated ATP hydrolytic activity, even measured at suboptimal pH's (Fenwick & Ma), exceeded reported Na^+/K^+-ATPase activity to such an extent that Ca^{2+}-transport rates would surpass expected Na^+-transport rates by several orders of magnitude. By the data presented in the literature the reverse is indicated (Fleming, 1973). Additionally, there has been no unqualified evidence published to indicate that ATP is the preferred substrate for the presumptive Ca^{2+}-ATPase. However, in higher vertebrates this Ca^{2+}-ATPase has been tentatively identified as an alkaline phosphatase-like activity, instead of an ATPase with characteristics of an ion transporting enzyme. In 1970, Haussler *et al.* (1970) were the first to report that alkaline phosphatase, Ca^{2+}-ATPase and much of the Mg^{2+}-ATPase activities of chick brush border membranes are properties of the same enzyme protein. More support for this idea was given by Oku & Wasserman (1978), who studied these activities in chick intestinal brush borders, and by Hanna *et al.* (1978, 1979) in their studies on these activities in rat enterocyte plasma membranes.

Recently, Ghijsen & Van Os (1979, 1982), Ghijsen *et al.* (1980, 1982), Van Os *et al.* (1980) and Van Os & Ghijsen (1981) showed in a series of studies on the mechanisms of calcium transport in the kidney cortex and gut of rats, that Ca^{2+}-activated ATPase activity is not homogeneous. To be more specific, they detected two different Ca^{2+}-stimulated ATP hydrolytic enzymic activities in the plasma membranes of enterocytes. One phosphatase was located on both the brush border

and the basolateral membranes, had a predominantly low affinity site for calcium, and was inhibited by theophylline and L-phenylalanine, both of which are specific inhibitors of alkaline phsphatase. The other phosphatase, which was located exclusively in the basolateral membranes, had high affinity for calcium, was not inhibited by alkaline phosphatase inhibitors, but was specifically inhibited by the calmodulin antagonist chlorpromazine. Additionally, they showed that the rate of accumulation of calcium by sealed vesicles of basolateral membranes was an exponent of the activity of high calcium affinity Ca^{2+}-ATPase and not of the low calcium affinity Ca^{2+}-ATPase. As a result of these studies they concluded that the low affinity phosphatase was an alkaline phosphatase-like enzyme and that the high affinity phosphatase was the true Ca^{2+}-ATPase with characteristics of an ion transporting enzyme. This is supported by the observation that (1,25-dihydroxy)vitamin-D_3 treated rachitic rats show concurrent increases in intestinal calcium absorption, high-affinity Ca^{2+}-ATPase activity and ATP-dependent Ca^{2+}-transport in the basolateral membranes.

Because of the difficulties listed earlier, and the recent evidence for the existence of at least two Ca^{2+}-dependent phosphatases, we decided to re-evaluate characteristics of the putative teleost gill Ca^{2+}-ATPase using more rigorous and inclusive criteria to investigate whether the low affinity non-specific phosphatase activity or the high affinity Ca^{2+}-ATPase activity is the most likely candidate for the energy generating source of the calcium pump in eel gills. To this end we improved the previously reported procedure (Fenwick, 1976) for the isolation of gill plasma membranes. Additionally, we prevented the ATPase activity which might have resulted from mitochondrial contamination by adding as a routine specific mitochondrial ATPase inhibitors to the assay media.

MATERIALS AND METHODS

Yellow female eels, *Anguilla rostrata* LeSueur, were used. The fish used for enzyme characterization studies had an average body weight of 1.7 kg and were obtained in the spring of 1982 from a commercial fish dealer in Québec City, Québec, Canada. The eels were held in running dechlorinated Ottawa city tap-water (0.45 mM Ca^{2+}, 12°C) under 16 h of light alternating with 8 h of darkness. During the experiments the fish were not fed. The experiments were carried out in the summer of 1982.

Isolation of plasma membranes

Animals were quickly anaesthetized in a Tris-buffered (pH 7.4) MS-222 solution (6 g/l). The bulbus arteriosus was cannulated and the branchial apparatus was perfused with ice cold isotonic saline containing heparin (20 U/ml) to remove the blood cells from the gills. Additionally, 0.2 mM phenylmethylsulphonyl-fluoride (PMSF), a protease inhibitor, was added to the perfusion fluid to increase enzyme recovery. The branchial epithelium was scraped off onto an ice-cold glass plate with a glass microscope slide. The subsequent rapid (3 h) preparative isolation procedure was carried out at 0-4°C. The collected material (± 2.5 g/kg eel) was disrupted gently with a douncer fitted with a loose pestle (20 strokes) in 15 ml of a hypotonic buffer (pH 8.0) containing (mM): NaCl (25), PMSF (0.2) and Hepes/Tris (1). The homogenate was diluted with the same buffer to 75 ml and centrifuged 15 min at 550 g. The pellet, containing nuclei and cellular debris, was discarded. The supernatant (H_0) was centrifuged for 30 min at 27 K rpm (Beckmann SW_{28} rotor) and yielded a pellet containing the membrane fraction (P_0). The pellet was resuspended with a douncer (100 strokes) in 15 ml of an isotonic buffer containing (mM): sucrose (250), NaCl (12.5), H_2EDTA (0.5), Hepes/Tris (5, pH: 7.5); osmolarity: 300 mOsm/l. H_2EDTA was added to ensure a leaky vesicle preparation. The suspension was diluted with the same buffer to 30 ml and centrifuged differentially: 1 Kg. 10 min, 10 Kg. 10 min, 30 Kg. 30 min. The final pellet (P_3) was used as the eel gill plasma membrane fraction. This pellet was resuspended with a douncer (100 strokes) in the basic assay buffer (mM): Tris-HCl (20), NaCl (100), pH, 7.4. To prevent cryodamage of membrane proteins, portions as required for the assays were quickly frozen in liquid nitrogen and stored at -90°C until use.

Assays and assay media

Protein. Membrane protein was estimated with a commercial reagent kit (BioRad) using bovine serum albumin as reference.

Na^+/K^+-ATPase. Na^+/K^+-ATPase was assayed by the method of Bonting & Caravaggio (1963). The incubation was started by the addition of a 20 µl sample of the membrane preparation, containing 10-20 µg protein, to 400 µl medium. The ouabain-sensitive, K^+-dependent activity was calculated as the difference in activities measured in two media. Medium A, yielding total ATPase activity, consisted of (mM): NaCl (100), $MgCl_2$ (5), H_2EDTA (0.1), Na_2ATP (3), KCl (12.5) and imidazole (30); pH 7.4. Medium E, yielding the ouabain-insensitive, K^+-independent activity, consisted of medium A without KCl but with added ouabain (1.0 mM). The incubation

was performed at 37°C for 10-30 min. The reaction was stopped on ice and by adding 100 µl ice-cold TCA (40%). To determine ATP hydrolysis, the liberated P_i was estimated as described by Fenwick (1976) with a Technicon auto-analyzer.

Alkaline phosphatase (pH 10.4)

Membrane alkaline phosphatase was estimated with a commercial reagent kit (Sigma) for the assay of serum or plasma alkaline phosphatase: *p*-nitrophenyl-phosphatase (*p*-NPPase) activity was assayed at pH 10.4 in a glycine buffer in the presence of 5 mM Mg^{2+}. Eel gut alkaline phosphatase (Sigma, type XIX) served as a reference. Activities were expressed in units eel gut alkaline phosphatase equivalents on basis of *p*-NP release. After the reaction was stopped with 1 N NaOH, *p*-NP was measured at 420 nm.

Succinate dehydrogenase

Succinate dehydrogenase (SDH) activity was estimated in a medium (pH 8.0) containing (mM): K_2HPO_4/KH_2PO_4 (50), Na_2-succinate (50), sucrose (25), and 1 mg/ml 2-(*p*-iodophenyl)3-(*p*-nitrophenyl)-5-phenyltetrazoliumhydrochloride(*p*-INT) according to Pennington (1961). The reaction was started by the addition of samples varying from 20-100 µl to 1 ml assay medium and incubations were carried out at room temperature until appropriate pink coloration. SDH activities were determined on the same day as isolation. The reaction was stopped by the addition of 100 µl TCA (40%) and extraction was done with 4 ml ethylacetate. The activity was determined in the organic phase by measuring the absorbance at 490 nm after overnight phase separation at 4°C. Activities were expressed as ΔA_{490}/h.mg protein.

Ca^{2+}-stimulated phosphatases

The basic medium for Ca^{2+}-stimulated phosphatase activities (pH 7.4) consisted of (mM): NaCl (100), ouabain (0.1), NaN_3 (5), oligomycin B (5 µg/ml), Tris-HCl (20); disodium salts of ATP, ADP, AMP and *p*-NPP were used as substrates, $Ca^{2+}\sim$ or $Mg^{2+}\sim$ substrate complexes were prepared by adding equimolar concentrations of the chloride salts of either ion, assuming a 1:1 ratio for $Me^{2+}\sim$ chelation by adenosinephosphate-esters (Sapper *et al.*, 1980). Incubations were performed on 20 µl samples in a total volume of 500 µl for 30 min at 25 or 37°C. Substrate hydrolysis was estimated as described for the Na^+/K^+-ATPase assay.

In all assays blanks were prepared by adding membrane samples after the reaction was stopped.

Lipophilic inhibitors (chlorpromazine, phenothiazine, R24571) were dissolved in ethanol (100%) and brought to the required concentration in the assay medium (ethanol concentrations did not exceed 0.1%). Membrane samples were preincubated with the desired final concentration of these inhibitors for 15 min at 37°C as suggested for R24571 by Gietzen *et al.* (1981). Ethanol treated samples served as controls. All assays were performed in plastic tubes.

Water soluble inhibitors (theophylline, 1-phenylalanine, cysteine) were directly dissolved in the assay medium.

Reagents

All reagents used were of the highest purity commercially available. Ultrapure water was used in all assays. R24571 was purchased from Janssen Pharmaceutica, Beerse, Belgium. All other chemicals were obtained from Sigma (St. Louis, MO).

Statistics and calculations

Values are expressed as mean values ± S.E.M. Statistical analysis of the data was carried out applying Student's t-test. Significance was accepted with $P < 0.05$ when $n \leq 4$, or with $P < 0.02$ when $n > 4$ (α=5%). Apparent K_m-values and V_{max}-values were calculated by means of Lineweaver-Burk transformation of the Michaelis-Menten equation. Linear regression analysis was based on the least squares method.

RESULTS

Isolation procedure

Table 1 shows the percentage recovery and the purification factors for several marker enzymes in the P_3-fraction. Na^+/K^+-ATPase was used as plasma membrane marker, while succinate dehydrogenase was taken as a marker for mitochondrial membranes. The P_3-fraction contained about 13% of the initial Na^+/K^+-ATPase and underwent a 60-fold purification. Succinate dehydrogenase activity was purified to only a factor of about 1.6 and showed a recovery of about 3.4%. Although a high degree of purification for plasma membranes was obtained as judged by the Na^+/K^+-ATPase purification factor, the P_3-fraction still showed mitochondrial ATPase activities. Only around 2% of the initial alkaline phosphatase (measured

Table 1. Recovery and purification of eel gill plasma membranes (P_3-fractions).

Recoveries were expressed as percentages of the total enzyme activities in the initial homogenate (H_0). The purification factors represent the ratios of the specific activities in the plasma membrane fraction (P_3) and the initial homogenate (H_0). Mean values (± S.E.M.) are given, with the number of observations in parentheses.

	Na^+/K^+-ATPase	Alkaline phosphatase	Succinate dehydrogenase	Protein
Recovery (%)	13.1 ± 2.33 (6)	1.8 ± 0.76 (6)	3.39 ± 0.39 (7)	2.6 ± 0.57 (7)
Purification	61.9 ± 5.76	0.91 ± 0.21	1.62 ± 0.36	-

at pH 10.4) was recovered with a purification factor of 0.91. The use of alkaline phosphatase as plasma membrane marker was suggested by Ma *et al.* (1974) but we conclude this enzyme to be inappropriate in the present study. We prefer the use of Na^+/K^+-ATPase as a plasma membrane marker because of its membrane-specificity and its specific ouabain-sensitivity.

Test for substrate accessibility of the membrane vesicles

Tween-80 had no significant effect on Na^+/K^+-ATPase activity of membrane vesicles of the P_3-fraction (Table 2). As detergent treatment of sealed vesicles should increase Na^+/K^+-ATPase activity (Brotherus *et al.*, 1979) as a result of improved substrate accessibility, we concluded that the membrane vesicles of the P_3-fraction were sufficiently leaky and that they did not require detergent treatment for optimal substrate- and ion-accessibility. Apparently the use of EDTA in the isolation procedure secures optimal enzyme activities in this plasma membrane vesicle preparation.

Table 2. Effects of Tween-80 on the Na^+/K^+-ATPase activities in eel gill plasma membranes (P_3-fractions).

Na^+/K^+-ATPase activities were used as an indicator of substrate accessibility of the membrane vesicle preparation. Samples were preincubated with Tween-80 (0.1% v/v) at 37°C for 10 min and subsequently incubated for 15 min at 37°C.

Similar, buffer preincubated, samples served as controls. Mean values (± S.E.M.) are given for six different samples, with the percentage activity in parentheses.

	Na^+/K^+-ATPase (μmol P_i/h/mg protein)	
	Controls	Detergent treated
Tween-80	51.25 ± 14.1 (100%)	52.14 ± 15.4 (103%)

Effects of storage on Ca^{2+}- or Mg^{2+}-activated ATP hydrolysis

In Fig. 1 the effects of storage at -90°C on the specific activities of Ca^{2+}- or Mg^{2+}-activated ATP hydrolysis is presented. The Ca^{2+}-induced ATP hydrolytic activity proved to be more stable than the Mg^{2+}-induced ATP hydrolytic activity with 61 and 37% of the original activity, respectively, being present after eight days. As a result of the difference in sensitivity towards storage at low temperature, the ratio of Mg^{2+}-induced ATP hydrolysis to Ca^{2+}-induced ATP hydrolysis decreased from 0.86 to 0.61 after one day and to 0.53 after 8 days ($P < 0.05$). Both curves showed the characteristics of a curve composed of a fast and a slow component. A rapid decrease is observed during the first day of storage, followed by a slower decrease in activity thereafter. Comparable results were presented by Ma *et al.* (1974) who stored their gill membrane preparations at -20°C.

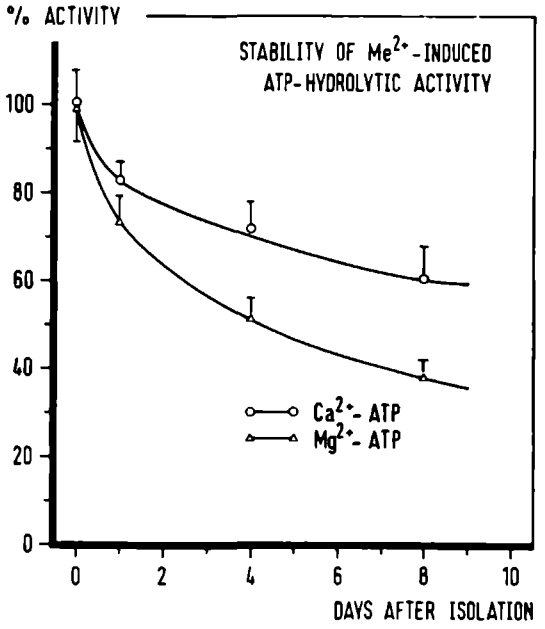


Figure 1. Effects of storage on Mg^{2+}-induced ATP hydrolytic activities in eel gill plasma membranes. Aliquots of P_3-fractions were frozen in liquid nitrogen and stored at -90°C. Me^{2+}-induced ATP hydrolysis (at 3 mM Mg∿ATP or Ca∿ATP) was assayed on the day of isolation and 1, 4 and 8 days afterwards, as described in Materials and Methods. Specific activities at day 0 were designated 100% activity and amounted to 52.8 ± 7.2 µmol/P_i/h/mg protein for Ca∿ATP and 45.4 ± 8.9 µmol/P_i/h/mg protein for Mg∿ATP. Mean values and standard errors of the mean are given; n=4.

It is tempting to suggest that the difference between Ca^{2+}- and Mg^{2+}-induced ATP hydrolysis in sensitivity to storage was a result of the differential inactivation of a heterogeneous enzyme pool present in eel gill plasma membranes.

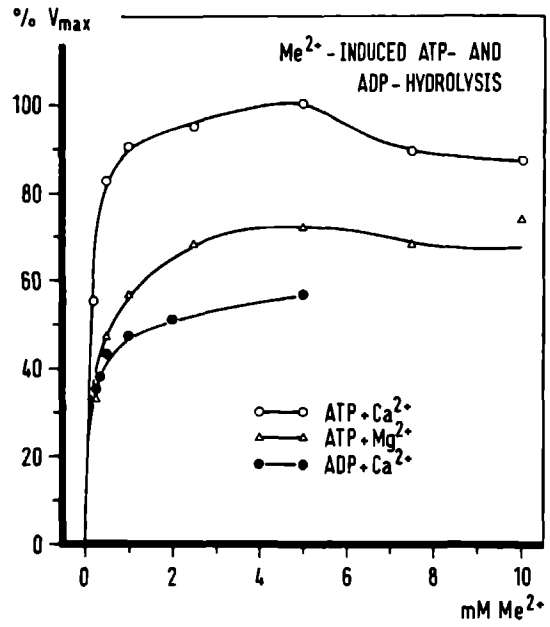
Activation of adenosinephosphate-ester hydrolysis by Ca^{2+}- or Mg^{2+}-ions

Significant hydrolysis of adenosinephosphate-esters upon incubation with eel gill plasma membranes was strictly dependent on the presence of Ca^{2+}- or Mg^{2+}-ions. This requirement for hydrolytic activity may involve activation of enzymes or a modification of the conformation of the substrates due to the chelation of these ions to adenosine phosphate-esters. The high concentrations of the ions required for maximum activation (5 mM Ca^{2+} + 5 mM ATP; Fig. 2) favour the latter explanation.

In a solution with a mixture of ATP, ADP and AMP and non-saturating concentrations of Ca^{2+}- or Mg^{2+}-ions, these ions will preferentially be chelated with the most complex ligand (ATP) because the affinity of the adenosinephosphate-esters for Ca^{2+}- or Mg^{2+}-ions increases with increasing numbers of phosphate groups in these esters (Table 3). At equimolar concentrations of Ca^{2+} and ATP, enzymatic breakdown of the Ca^{2+}∿ATP complex will give Ca^{2+}∿ADP and phosphate as reaction products. If, on the other hand, a surplus of ATP is present relative to Ca^{2+}-ions, upon enzymatic hydrolysis of Ca^{2+}∿ATP the Ca^{2+}-ions will migrate from the reaction product Ca^{2+}∿ADP to the free ATP, which leads to a reaction mixture of Ca^{2+} ATP, and free ATP and ADP.

Fig. 2 shows the effects of different concentrations of Ca^{2+}- or MG^{2+}-ions on ATP or ADP hydrolysis at fixed concentrations of ATP or ADP (5 mM). Maximum activities consistently occurred at equimolar concentrations of divalent ion and ATP or ADP. The V_{max}-value for Mg^{2+}-activation of ATP hydrolysis was 71.5% that of the V_{max}-value found for Ca^{2+}-activation of ATP hydrolysis.

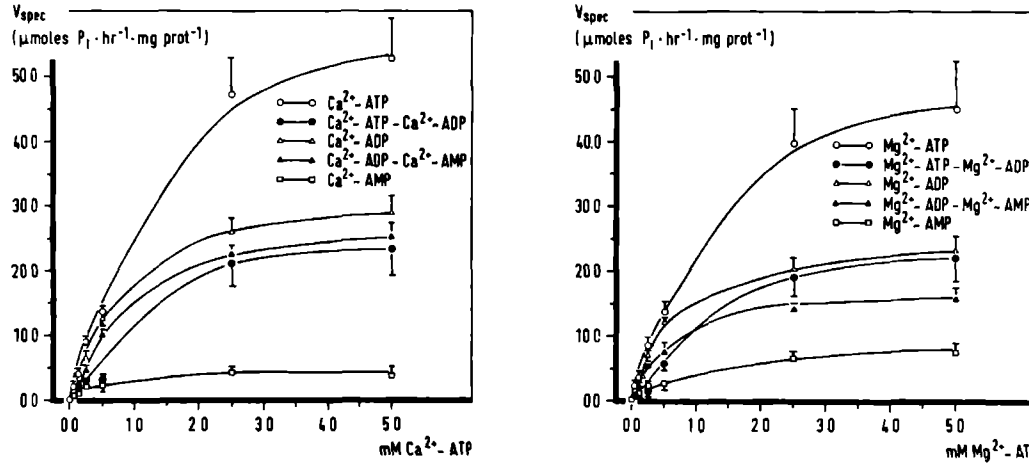
Figure 2. Effects of different concentrations of Ca^{2+}- or Mg^{2+}-ions on ATP and ADP hydrolysis by eel gill plasma membranes (P_3-fraction). Activities were expressed as percentages of the maximum hydrolytic activity (observed at 5 mM Ca^{2+} and 5 mM ATP). Specific activities amounted to 60.2 ± 2.8 µmol P_i/h/mg protein for 5 mM Ca∿ATP, 34.7 ± 8.1 µmol P_i/h/mg protein for 5 mM Ca∿ADP and 43.1 ± 3.9 µmol P_i/h/mg protein for 5 mM Mg∿ATP. Mg^{2+}-concentrations that resulted in half-maximal activation of substrate hydrolysis (calculated with Lineweaver-Burk plots): 78 µM Ca^{2+} for ATP, 51 µM Ca^{2+} for ADP and 101 µM Mg^{2+} for ATP. Mean values of six observations are given.



The V_{max}-values for Ca^{2+}-activation of ADP Hydrolysis came to 57% of the V_{max}-values for Ca^{2+}-activation of ATP hydrolysis. The apparent K_m-values for Mg^{2+}- or Ca^{2+}-induced substrate hydrolysis were 78 µM Ca^{2+} for ATP, 51 µM Ca^{2+} for ADP and 101 µM Mg^{2+} for ATP. ATP-hydrolysis was increasingly diminished at Mg^{2+}- or Ca^{2+}-concentrations exceeding the ATP concentrations (2.5 or 5.0 mM).

In Figs. 3(a) and (b) and Table 4 the results of substrate-specificity determinations are shown. Apparent K_m-values and V_{max}-values were calculated on the

Figure 3. Eel gill phosphatase substrate specificity tests. Aliquots of P_3-fractions were incubated with equimolar concentrations of adenosine-phosphate esters and either Ca^{2+} (left) or Mg^{2+} (right). Four plasma membrane preparations were tested with six different substrates each. Additionally, curves are shown that represent the difference between the specific activities measured with ATP and ADP, and the difference between the specific activities measured with ADP and AMP. Half-maximal activation concentrations and maximum velocities (calculated with Lineweaver-Burk plots) are given in Table 4. Mean values and standard errors of the mean are given; n=4.



basis of incubations of plasma membranes with varying concentrations of equimolar amounts of phosphate-esters (ATP, ADP, AMP or p-NPP) and divalent ions (Ca^{2+} or Mg^{2+}). V_{max}-values for ADP as compared to ATP (=100%) are 55 and 51% for Ca^{2+} and Mg^{2+} respectively. The V_{max}-values for Mg∿AMP were twice as high as observed for Ca∿AMP or p-NPP in combination with either Ca^{2+} or Mg^{2+}.

Apparent K_m-values decreased with decreasing numbers of phosphate groups in the substrate, i.e. the apparent affinities for the simples substrates (AMP and

Table 4. Apparent K_m-values and V_{max}-values for Ca^{2+}- and Mg^{2+}-substrate complexes in eel gill plasma membranes.

K_m-values (mM) and V_{max}-values (µmol P_i/h/mg protein) were calculated for individual samples by means of Lineweaver-Burk plots. Calculated V_{max}-values were compared with observed V_{max}-values. Mean values are given (± S.E.M.) for four different samples. Significance of differences is given in the text.

Substrate	Apparent K_m	V_{max} Calculated	Observed
Ca∿ATP	0.83 ± 0.13	38.16 ± 4.67	52.78 ± 7.24 (100%)
Ca∿ADP	0.53 ± 0.13	23.95 ± 5.43	28.93 ± 2.63 (55%)
Ca∿AMP	0.10 ± 0.04	3.43 ± 0.53	3.69 ± 0.30 (7%)
Ca-p-NPP	0.14	2.76	2.76 ± 0.12 (5%)
Mg∿ATP	0.52 ± 0.10	29.03 ± 7.43	45.42 ± 8.85 (100%)
Mg∿ADP	0.38 ± 0.12	20.12 ± 3.55	23.39 ± 2.33 (51%)
Mg∿AMP	0.20 ± 0.07	5.34 ± 1.07	7.35 ± 0.86 (16%)
Mg-p-NPP	0.14	3.03	3.03 ± 0.64 (7%)

p-NPP) were found to be the highest. No difference was found in apparent affinity between AMP or p-NPP in combination with either Ca^{2+} or Mg^{2+}. The apparent affinity for Mg∿ATP and Mg∿ADP was significantly higher than for Ca∿ATP or Ca∿ADP.

For the ATP complexes as substrates the calculated and observed V_{max}-values differed significantly. This suggested that the results did not fulfil the requirements for a Lineweaver-Burk transformation. This again may indicate that the enzyme preparation is heterogeneous. On the basis of the apparent K_m-values in Table 4 it may be expected that, when ATP-complexes are given as substrate, the reaction product Ca∿ADP will preferentially be hydrolyzed to Ca∿AMP and subsequently Ca∿AMP to adenosine. As the release of phosphate was measured as an indication for substrate hydrolysis, the V_{max}-values were very likely overestimated when ATP or ADP were used as substrates. Therefore, the saturation curves for ATP and ADP hydrolysis were also presented as the differences between ATP and ADP hydrolysis and between ADP and AMP hydrolysis. The differences between the corrected values for V_{max} of ATP and ADP hydrolysis are not significant.

From these observations we conclude that under the conditions described no high affinity Ca^{2+}-ATPase can be demonstrated. Instead the observed hydrolytic

activities represent non-specific phosphatase activities. We therefore examined the effects of several inhibitors on Ca∿ATP and Mg∿ATP hydrolysis in another set of experiments.

Effects of inhibitors on Ca∿ATP and Mg∿ATP hydrolysis (Table 5)

Under our assay conditions, up to 10 mM L-phenylalanine (a known inhibitor of gut alkaline phosphatase) had no inhibitory effect on either Ca∿ATP or Mg∿ATP hydrolysis. Conversely, theophylline, a more potent inhibitor of alkaline phosphatase (Ghijsen *et al.*, 1980), inhibited significantly more Ca∿ATP hydrolysis than Mg∿ATP hydrolysis (23.4 and 14.6% respectively). Both activities were inhibited by up to 80% with L-cysteine (10 mM). However, the calculated 50% inhibition occurred at 2.27 mM cysteine for Ca∿ATP hydrolysis and 6.08 mM cysteine for Mg∿ATP hydrolysis (Fig. 4).

Table 5. Effects of various inhibitors on Ca∿ATP and Mg∿ATP hydrolysis in eel gill plasma membranes.

Inhibition (%) at the most effective inhibitor concentration (mM) is given for the average of six individual samples tested with and without inhibitors.

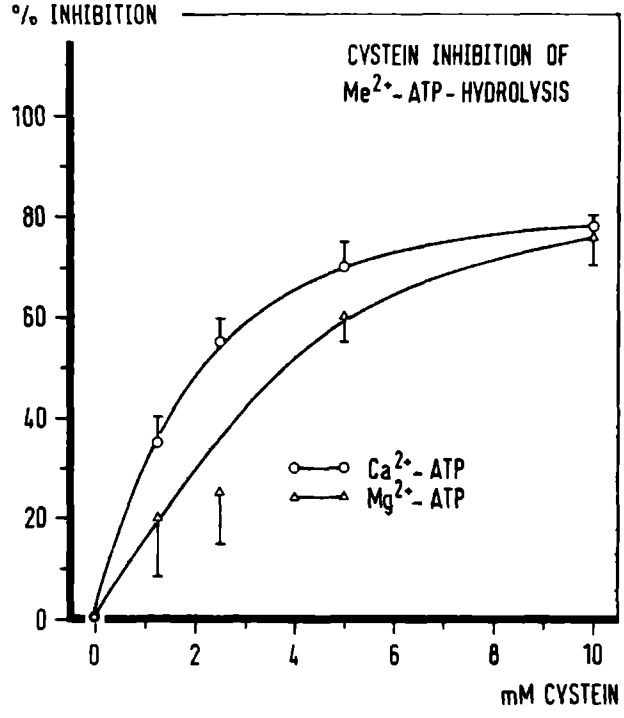
	Maximum inhibition (%)	
	Ca∿ATP	Mg∿ATP
Inhibitor	(3 mM)	(3 mM)
L-Phenylalanine (10.0)	0.0	0.0
Theophylline (1.25)	23.4	14.6
Cysteine (10)	77.2	76.7
R24571 (0.01)	5.1	14.1
Chlorpromazine (0.1)	6.1	9.0
Phenothiazine (0.001)	6.0	10.8

R24571, chlorpromazin and phenothiazin have been reported to inhibit calcium transport related high-affinity Ca^{2+}-ATPase activities by competition with calmodulin at very low inhibitor concentrations (0.01-1.0 µM) or by affecting the calmodulin-independent basal activities at higher concentrations (1.0-10.0 µM). Maximum inhibition of Ca∿ATP or Mg∿ATP hydrolysis occurred at the higher inhibi-

tor concentrations but no significant differences were observed between the effects of these inhibitors at low or high concentrations. Mg∿ATP hydrolysis was, however, significantly more sensitive (14% inhibition) towards these inhibitors than Ca∿ATP hydrolysis (6% inhibition).

These results confirm that the Ca∿ATP or Mg∿ATP hydrolytic activities in eel gill plasma membranes are not homogeneous.

Figure 4. L-Cysteine inhibition of Mg∿ATP and Ca∿ATP hydrolysis by eel gill plasma membranes (P_3-fraction). Half-maximal inhibition was calculated to occur at 2.2 mM L-cysteine for Ca∿ATP and at 5 mM L-cysteine for Mg∿ATP. Specific activities in the absence of L-cysteine amounted to 38.2 ± 4.7 µmol P_i/h/mg protein for Ca∿ATP (3 mM) and 23.3 ± 3.0 µmol P_i/h/mg protein for Mg∿ATP (3 mM). Mean values and standard errors of the mean are given; n=6.



DISCUSSION

Four major findings are presented in this paper. (1) Ca^{2+}-activated ATPase activities occurring in the gills of American eels are located in the plasma membranes of this epithelium. (2) The assay procedures for fish gill Ca^{2+}ATPase previously described in the literature, and retested in this study did not allow the determination of a single, specific Ca^{2+}-ATPase. (3) Eel gill plasma membranes contain more than one type of enzyme that hydrolyzes complexes of Ca^{2+}- or Mg^{2+}-ions and adenosinephosphate-esters, as indicated by substrate specificity tests and effects of various inhibitors on these hydrolytic activities. (4) These Ca^{2+}-activated ATPase activities do not represent high affinity, transport Ca^{2+}-ATPase activity but rather non-specific "alkaline" phosphatase activities.

Location of Ca^{2+}- or Mg^{2+}-activated ATPase activities

The procedure used for isolating the plasma membranes of eel gills in this study yielded a highly enriched plasma membrane fraction, as indicated by the Na^{+}/K^{+}-ATPase specific activities, with only minor mitochondrial contamination. To exclude any interference of mitochondrial ATPase activities, oligomycin and sodium azide were routinely added to the assay media. We therefore conclude that the observed Ca^{2+}- or Mg^{2+}-activated ATPase activities reside in the plasma membranes of the gill epithelium. But we do acknowledge that a portion of these activities may have originated from enzyme activities of endoplasmatic reticulum origin as we did not screen for marker enzyme activities for these membranes during the isolation procedure. The activation curves observed for ATP hydrolysis at increasing concentrations of Ca^{2+}- or Mg^{2+}-ions with this preparation resembled closely those previously reported for gill membranes of rainbow trout (Ma *et al.*, 1974), American eels (Fenwick, 1976), roach (Shepard, 1981), and tilapia (our unpublished observations). The calculated K_m-values for Ca^{2+}- or Mg^{2+}-activation of ATP hydrolysis are similar to previously published values (Fenwick, 1979). The differences in V_{max}-values presented here and those in the earlier literature may be the result of the use of more highly purified membrane fractions in the present study and from differences in incubation temperatures.

Characteristics of the Ca^{2+}-activated ATPase activities

One of the results of this study we wish to emphasize is that the assay proce-

dures previously employed for Ca^{2+}-activated ATPase activities in fish gills (among others) did not fulfil the requirements for the determination of transport Ca^{2+}-ATPases. Rather they yielded non-specific activities of Ca∿ATP or Mg∿ATP hydrolyzing enzymes. To support this contention we propose four major criticisms. (1) The affinity of the reported ATPase for Ca^{2+} was too low for an enzyme that must be stimulated by Ca^{2+} at intracellular Ca^{2+}-concentrations. (2) This Ca^{2+}-stimulated ATP hydrolysis, even measured at suboptimal pH (Fenwick, 1979), would indicate Ca^{2+}-transport rates that would surpass the Na^{+}-transport rates by several orders of magnitude. (3) No evidence was provided that ATP was the preferential substrate. (4) The pH-optima for the activities were reportedly in the alkaline trajectory (Ma *et al.*, 1974; Fenwick, 1976) and were thus characteristic for alkaline phosphatase.

Because of these criticisms we conclude that the observed activation curves for Ca^{2+}- or Mg^{2+}-induced ATP hydrolysis represent saturation curves for complexes of ATP with either metal ion rather than Ca^{2+}- or Mg^{2+}-activation of ATP-hydrolyzing enzymes. Hydrolysis of ATP is strictly dependent on the presence of Ca^{2+} or Mg^{2+}, suggesting that ATP alone is not (detectably) used as a substrate. Upon addition of Ca^{2+} or Mg^{2+} to ATP-containing media, these metal ions will be chelated by ATP (Walaas, 1958; Sapper *et al.*, 1980). Although Mg^{2+} is chelated more strongly than Ca^{2+} by ATP, both interactions have the same modifying effect on the conformation of the adenosine-phosphate-ester and thus allow effective binding of the phosphate group to the active site of enzymes. In this context it should be mentioned that Na^{+}/K^{+}-ATPase (Bonting & Caravaggio, 1963) as well as the typical transport Ca^{2+}-ATPases of rat intestine (Ghijsen *et al.*, 1980) are dependent on Mg∿ATP although they may be inhibited by Ca∿ATP (Epstein & Whittam, 1966).

Maximum velocities are found at equimolar concentrations of Ca^{2+} or Mg^{2+} and ATP or ADP. However, when Ca^{2+}- or Mg^{2+}-ion concentrations exceeded the ATP-concentration by 2.5 or 5.0 mM, ATP-hydrolysis was inhibited. This indicates that Ca-ATP and Mg-ATP hydrolysis is inhibited by high levels of free Ca^{2+} or Mg^{2+} respectively.

The possibility cannot be excluded that in our assay procedure, stimulation of free or complexed ATP hydrolysis occurs by micromolar concentrations of free Ca^{2+} or Mg^{2+}. But if we assume that the presence of both Ca^{2+} and Mg^{2+} in the assay medium is a prerequisite for the determination of a transport Ca^{2+}-ATPase, it is clearly not possible to show such an enzyme with the assay procedures applied in this and many other studies. Additionally, we want to stress that free Ca^{2+}-ion concentrations of the assay media in the micromolar range cannot

be obtained reliably unless appropriate buffering with EGTA or NTA is employed (Sharff, 1979, 1981; Ghijsen *et al.*, 1980).

Moreover, when a surplus of ATP relative to Ca^{2+} is used, new substrate will constantly be produced in the assay medium as the free ATP will react with the reaction product Ca-ADP to produce Ca-ATP and ADP. When this Ca^{2+}-dependent substrate production occurs, the activation curve represents the effects of Ca^{2+}-defined substrate concentrations rather than the effects of Ca^{2+}-concentrations on ATP-hydrolytic activities. When ADP was substituted for ATP in this type of experiment, comparable results were obtained. No phosphate release occurred in the absence of Ca^{2+}. The shape of the activation curve observed resembled the one for ATP and suggested that the hydrolysis of ADP is also dependent on the chelation of Ca^{2+}.

As P_i-release was measured as an indication of substrate hydrolysis in all cases, no straightforward answer can be given as to whether these activities were the result of the hydrolysis of a single substrate-complex or of a combination of the original substrate and the reaction products. K_m-values calculated for Ca^{2+}-activation of ATP or ADP hydrolysis by the enzyme preparations used, may therefore be incorrect due to the continuous production of new substrate. If such was the case the affinities for complexes of ATP and ADP with Ca^{2+} or Mg^{2+} were probably overestimated.

To test the preferences for Ca^{2+}- and Mg^{2+}-substrate complexes we measured the activation curves for these complexes, assuming a 1:1 ratio for Ca^{2+}- or Mg^{2+}-chelation by the adenosinephosphate-esters, K_m-values were found to be much higher than the values found in the presence of unchelated ATP, probably because substrate concentrations become limiting at low concentrations. Surprisingly, K_m-values calculated from Lineweaver-Burk plots for the different complexes were highest for the ATP-complexes and lowest for the AMP-complexes. As the affinity for ADP and AMP, which appear as reaction products during the hydrolysis of ATP, were higher than for the original ATP it was to be expected that hydrolysis of the first-given substrate would continue until dephosphorylation was complete. Therefore the V_{max}-values for ATP- and ADP-complexes calculated on the basis of P_i-release are overestimated. Thus when P_i-release is used as a measure for substrate hydrolysis, the rate of ATP hydrolysis is better estimated as the difference between ATP and ADP hydrolysis, and ADP hydrolysis as the difference between ADP and AMP hydrolysis. Corrected in this way, the V_{max}-values for Ca∿ATP, Ca∿ADP and Mg∿ATP were not significantly different from each other. The values for Mg∿ADP were significantly lower; those for Mg∿AMP and Ca∿AMP were lower still. This would clearly point again to the non-specific character of these phosphatase activities.

However, care should be taken with results obtained from Lineweaver-Burk plots when complicated samples such as plasma membrane-preparations are used. When the same results are plotted according to Eadie-Hofstee, no straight lines were observed for any substrate tested. This would mean then that the data do not fulfil the requirements for kinetic analysis by means of a Lineweaver-Burk plot (Borst Pauwels, 1973). This finding might also explain why the calculated V_{max}-values were found to be lower than the observed values. Furthermore, the fact that no straight lines were observed in Eadie-Hofstee plots for substrate-complexes supports again the postulate that more than one phosphatase activity is present in eel gill plasma membranes. Another complicating factor is the observation that the high P_i-levels, which were yielded with high concentrations of substrate-complexes, may have inhibited phosphatase activities. This would result in an underestimation of V_{max}-values.

We conclude that the non-specific Ca∿ATP-phosphatase activity in the eel gill hydrolyzed Ca∿ADP and Mg∿ATP equally well (equal V_{max}-values), when substrate hydrolysis is estimated properly. These data are also more consistent with the thesis that the activation of ATP or ADP hydrolysis by Ca^{2+} or Mg^{2+} is more likely a result of chelation of these metal ions by the substrates rather than a direct stimulation of enzymes by Ca^{2+} or Mg^{2+}. But it cannot be excluded that the P_i measured when Ca∿ATP is the substrate, originates from a Ca∿ATP, an ATP hydrolyzing activity (or both) after stimulation by micromolar concentrations of free Ca^{2+} present in the reaction media. For the determination of such "high-affinity" Ca^{2+}-ATPases the assay media must contain Ca^{2+}-buffers to establish reliably micromolar concentrations of Ca^{2+}.

Effects of various inhibitors on Ca∿ATP and Mg∿ATP hydrolysis

As the previously reported pH-optima for Ca^{2+}-activated ATPase activities approximated a value of 8.0 (Ma *et al.*, 1974; Fenwick, 1976) we deduced that most of the P_i-release was due to non-specific alkaline phosphatases and we therefore tested the effects of various inhibitors on Ca∿ATP and Mg∿ATP hydrolysis. L-Phenylalanine, theophylline and L-cysteine are reported to affect non-specific phosphatases with alkaline pH-optima. Chlorpromazine, phenothiazine and calmidazolium (R24571) are reported to act as calmodulin-antagonists in a concentration-dependent way and to inhibit calcium transport in rat enterocytes and human erythrocytes by inhibition of the high-affinity transport Ca^{2+}-ATPases (Ghijsen *et al.*, 1982; Gietzen *et al.*, 1981).

L-Phenylalanine (up to 10 mM) did not affect Ca∿ATP or Mg∿ATP hydrolysis under

our standard conditions (pH 7.4). These results concur with the results of Ghosh & Fishman(1966), who similarly reported that phosphatase activity was not inhibited by L-phenylalanine at pH-values below 7.8. On the other hand, Ghijsen *et al.* (1980) did report significant L-phenylalanine-induced inhibition of alkaline phosphatase activity in rat enterocyte plasma membranes, at a pH of 7.4. This discrepancy may reflect the organ-specificity of L-phenylalanine suggested by Ghosh & Fishman (1966). Other explanations for this discrepancy are that the kind of substrate used was not appropriate to show the effects of the inhibitor, or the fact that membrane preparations were used instead of pure enzymes. Theophylline, a more potent inhibitor of alkaline phosphatase activities than L-phenylalanine, had maximal effects at a concentration of 1.25 mM on both Ca∿ATP and Mg∿ATP hydrolysis, but the Ca∿ATP hydrolysis was inhibited to a greater extent than the Mg∿ATP hydrolysis. This suggests that Ca∿ATP was the preferred substrate for a gill phosphatase activity. However, both Ca∿ATP and Mg∿ATP can be hydrolyzed by theophylline-sensitive enzyme activities. Maximum inhibition produced by theophylline was 20% of the total hydrolytic activity indicating again heterogeneity of enzyme activity. Cysteine, an alkaline phosphatase inhibitor that acts by binding the intrinsic Zn^{2+}-ion gave up to 80% inhibition of both Ca∿ATP and Mg∿ATP hydrolysis. Calculated I_{50}-values, however, differed significantly for these substrates: I_{50}-Ca∿ATP: 5.0 mM, I_{50}-Mg∿ATP: 2.2 mM. Thus, the cysteine would seem to act by dissociation of the substrate-complexes rather than directly via the enzymes. Therefore, these results do not allow the conclusion that the phosphate release is due to the presence of Zn^{2+}-containing enzymes.

The phenothiazines inhibited Mg∿ATP and Ca∿ATP hydrolysis to a different extent: maxima were only 6% for Ca∿ATP and 15% for Mg∿ATP. This finding suggests that a large quantity of non-specific phenothiazine insensitive phosphatase activity was present. But whether the effects of these inhibitors at high concentrations resulted from competition with calmodulins, which are abundantly present in these membrane preparations (unpublished observation) or resulted from an effect on membrane integrity, causing reduced hydrolytic activity (Van Belle, 1981), remains to be elucidated.

Tentatively, we conclude that at least a portion of the Ca∿ATP and Mg∿ATP hydrolytic activity is calmodulin dependent. This calmodulin-sensitive component preferentially hydrolyzes Mg∿ATP. However, the calmodulin-antagonists attenuate Ca^{2+}-activated ATPase activity only to a small extent. The latter conclusion indicates that a major part of the presumptive Ca^{2+}-ATPase activity cannot be involved in active Ca^{2+}-transport.

In further studies on Ca^{2+}-stimulated phosphatases in eel gill plasma mem-

branes we were able to discriminate between non-specific, Ca^{2+}-stimulated phosphatases and high-affinity Ca^{2+}-ATPase activity that may represent the calcium pump (data to be published separately). In fresh water adapted eels, both activities are correlated negatively with the environmental Ca^{2+}-concentration. Although the non-specific Ca^{2+}-stimulated phosphatase activity does not fulfil the requirements of a Ca^{2+}-transport ATPase, some relation of this activity with Ca^{2+}-transport phenomena seems to exist. Thus, more than one enzyme activity located in the plasma membrane may be involved in gill transepithelial Ca^{2+}-transport.

REFERENCES

BERG, A. (1968) Studies on the metabolism of calcium and strontium in fresh water fish. I. Relative contribution of direct and intestinal absorption. Me.Inst. Ital.Idrobiol. 23: 161-196.

BONTING, S.L. & CARAVAGGIO, L.L. (1963) Studies on Na^+/K^+-activated ATPase. V. Correlation of enzyme activity with cation flux in tissues. Archs Biochem. Biophys. 101: 37-717.

BORST PAUWELS, G.W.F.H. (1973) Two site-single carrier transport kinetics. J.theor.Biol. 40: 19-31.

BROTHERUS, J.R., JOST, P.C., GRIFFITH, O.H. & HOKIN, L.E. (1979) Detergent inactivation of sodium- and potassium-activated adenosinetriphosphatase of the electric eel. Biochemistry 18: 5043-5049.

DONEEN, B.A. (1981) Effects of adaptation to sea water, 170% sea water and to fresh water on activities and subcellular distribution of branchial Na^+/K^+-ATPase, low- and high-affinity Ca^{2+}-ATPase, and ouabain-insensitive ATPase in *Gillichthys mirabilis*. J.comp.Physiol. 145: 51-61.

EPSTEIN, F.H. & WHITTAM, R. (1966) The mode of inhibition by calcium of cell-membrane adenosinetriphosphatase activity. Biochem.J. 99: 232.

FENWICK, J.C. (1976) Effect of stanniectomy on calcium activated adenosinetriphosphatase activity in the gills of fresh water adapted North American eels, *Anguilla rostrata* LeSueur. Gen.comp.Endocr. 29: 383-387.

FENWICK, J.C. (1979) Ca^{2+}-activated adenosinetriphosphatase activity in the gills of fresh water- and sea water-adapted eels *(Anguilla rostrata)*. Comp.Biochem. Physiol. 62B: 67-70.

FLEMING, W.R. (1969) Electrolyte metabolism of teleosts-including calcified tissues. In Chemical Zoology (edited by Florkin, M. and Scheer, B.T.), Vol. VIII, 471-508. Academic Press, New York.

GHIJSEN, W.E.J.M. & VAN OS, C.H. (1979) Ca^{2+}-stimulated ATPase in brush border and basolateral membranes of rat duodenum with high affinity sites for Ca-ions. Nature, Lond. 279: 802-803.

GHIJSEN, W.E.J.M. & VAN OS, C.H. (1982) 1,25-Dihydroxy-vitamin D_3 regulates ATP-dependent calcium transport in basolateral plasma membranes of rat enterocytes. Biochim.Biophys.Acta 689: 170-172.

GHIJSEN, W.E.J.M., DE JONG, M.D. & VAN OS, C.H. (1980) Dissociation between Ca^{2+}-ATPase and alkaline phosphatase activities in plasma membranes of rat duodenum. Biochim.Biophys.Acta 599: 538-551.

GHIJSEN, W.E.J.M., DE JONG, M.D. & VAN OS, C.H. (1982) ATP-dependent calcium transport and its correlation with Ca^{2+}-ATPase activity in basolateral plasma membranes of rat duodenum. Biochim.Biophys.Acta 689: 327-336.

GHOSH, N.K. & FISHMAN, W.H. (1966) On the mechanism of inhibition of intestinal alkaline phosphatase by L-phenylalanine. J.biol.Chem. 241: 2516-2522.
GIETZEN, K., KONRAD, R., TEJCKA, M., FLEISCHER, S. & WOLF, H.U. (1981a) Purification, characterization, and reconstitution of the Ca^{2+}-transport system (high-affinity Ca^{2+}, Mg^{2+}-ATPase) of the human erythrocyte membrane. Acta biol.med.germ. 40: 443-456.
GIETZEN, K., WUTHRICH, A. & BADER, H. (1981b) R24571: a new powerful inhibitor of red blood cell Ca^{2+}-transport ATPase and of calmodulin-regulated functions. Biochem.Biophys.Res.Commun. 101: 418-425.
HANNA, S., MIRCHEFF, A. & WRIGHT, E. (1978) Purification of alkaline phosphatase and Ca^{2+}-ATPase from basal lateral membranes of rat duodenum. Biophys.J. 21: 203a.
HANNA, S.D., MIRCHEFF, A.K. & WRIGHT, E.M. (1979) Alkaline phosphatase of basal lateral and brush border plasma membranes from intestinal epithelium. J.supramol.Struct. 11: 451-466.
HAUSSLER, M.R., NAGODE, L.A. & RASMUSSEN, H. (1970) Induction of intestinal brush border alkaline phosphatase by vitamin D and identity with Ca^{2+}-ATPase. Nature, Lond.228: 1199-1201.
HO, S.M. & CHAN, D.K.O. (1980) Branchial ATPases and ionic transport in the eel *Anguilla japonica*-II. Ca^{2+}-ATPase. Comp.Biochem.Physiol. 67B: 639-645.
MA, S.W.Y. & COPP, D.H. (1978) Purification, properties and action of a glycopeptide from the corpuscles of Stannius which affect calcium metabolism in the teleost. In Comparative Endocrinology (edited by Gaillard, P.J. & Boer, H.H.), 283-286. Elsevier/North-Holland Biomedical Press, Amsterdam.
MA, S.W.Y., SHAMI, Y., MESSER, H.H. & COPP, D.H. (1974) Properties of Ca^{2+}-ATPase from the gill of rainbow trout *(Salmo gairdeneri)*. Biochim.Biophys.Acta 345: 243-251.
MASHIKO, K. & JOZUKA, K. (1964a) Absorption and excretion of calcium by teleost fishes with special reference to routes followed. Am.Zool.jap. 37: 41-50.
MOON, T.W. (1978) The characterization of ATPases from the gill of the osteoglossid *Osteoglossu bicirrhosum* (aruana). Can.J.Zool. 56: 795-800.
OKU, T. & WASSERMAN, R.H. (1978) Properties of Vitamin D-stimulated calcium-dependent adenosine triphosphatase (Ca^{2+}-ATPase) and alkaline phosphatase in chick intestinal brush border. Fed.Proc. 37: 408.
PENNINGTON, R.J. (1961) Biochemistry of dystrophic muscle (mitochondrial succinate tetrazolium reductase and adenosinetriphosphatase). Biochem.J. 80: 649-654.
SAPPER, H., GOHL, W. & LOHMANN, W. (1980) ^{31}P- und ^{1}H-NMR-Untersuchungen uber die Wechselwirkung von ATP mit Mg^{2+}, Ca^{2+} und Monoaminen. Z.Naturforsch. 35c: 569-577.
SHARFF, O. (1979) Comparison between measured and calculated concentrations of calcium ions in buffers. Analyt.Chim.Acta 109: 219-305.
SHARFF, O. (1981) Regulation of (Ca^{2+}, Mg^{2+})-ATPase in human erythrocytes dependent on calcium and calmodulin. Acta biol.med.germ. 40: 457-463.
SHEPARD, K.L. (1981) The activity and characteristics of the Ca^{2+}-ATPase of fish gills in relation to environmental calcium concentrations. J.exp.Biol. 90: 115-121.
SO, Y.P. & FENWICK, J.C. (1977) Relationship between net ^{45}calcium influx across a perfused isolated eel gill and the development of post-stanniectomy hypercalcaemia. J.exp.Zool. 200: 259-264.
VAN BELLE, H. (1981) R24571: a potent inhibitor of calmodulin-activated enzymes. Cell Calcium 2: 483-493.
VAN OS, C.H. & GHIJSEN, W.E.J.M. (1981) High affinity Ca-ATPase in basolateral plasma membranes of rat duodenum and kidney cortex. In Calcium and Phosphate Transport across Biomembranes (edited by Bronner, F. & Peterlik, M.), 159-162. Academic Press, New York.

VAN OS, C.H., DE JONGE, H.R., DE JONG, M.D., GHIJSEN, W.E.J.M. & WALTERS, J.A.L.I. (1980) Separation of basolateral plasma membranes from smooth endoplasmic reticulum of the rat enterocyte by zonal electrophoresis on density gradients. Biochim.Biophys.Acta 600: 730-738.

WALAAS, E. (1958) Stability constants of metal complexes with mononucleotides. Acta chem.scand. 12: 528-536.

WENDELAAR BONGA, S.E. & VAN DER MEIJ, J.C.A. (1980) The effect of ambient calcium on prolactin cell activity and plasma electrolytes in *Sarotherodon mossambicus (Tilapia mossambica)*. Gen.comp.endocr. 40: 391-401.

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Ca^{2+}-DEPENDENT PHOSPHATASE AND Ca^{2+}-DEPENDENT ATPase ACTIVITIES IN PLASMA MEMBRANES OF EEL GILL EPITHELIUM. II EVIDENCE FOR TRANSPORT HIGH-AFFINITY Ca^{2+}-ATPase.

ABSTRACT

1. Ca^{2+}-ATPase activity was studied in eel gill plasma membranes. In the presence of 2 mM free Mg^{2+}, Ca^{2+}-stimulated ATP- and ADP-hydrolysis was observed with up to 2 mM Ca^{2+}. Kinetic analysis of Ca^{2+}-induced substrate hydrolysis by Eadie-Hofstee plots suggested that the Ca^{2+}-ATPase activity consisted of two components in these membranes. One phosphatase component had high affinity for calcium ($K_{0.5}$: 0.22 µM, V_{max}: 5.41 ± 0.63 µmol $P_i.h^{-1}$.mg $protein^{-1}$) and a marked preference for ATP and was judged to be a typical high-affinity Ca^{2+}-ATPase. The second component had low affinity for Ca^{2+} ($K_{0.5} \geq 230$ µM) and hydrolyzed ATP and ADP equally well (V_{max}: 10.70 ± 1.25 µmol $P_i.h^{-1}$.mg $protein^{-1}$), characteristics consistent with non-specific phosphatase activities.
2. The high-affinity Ca^{2+}-ATPase activity, determined as the 1 µM Ca^{2+} induced ATP-hydrolysis in the presence of 2 mM Mg^{2+}_{free}, was inhibited about 75% by EGTA-treatment of the membrane preparation, 87% by 10^{-5}M chlorpromazine and 100% by 10^{-6}M calmidazolium (R24571). L-phenylalanine, theophylline, oligomycine or azide did not affect this high-affinity Ca^{2+}-ATPase activity.
3. Low-affinity phosphatase activity, determined at 200 µM Ca^{2+}, induced ATP- and ADP-hydrolysis with equal effectiveness and was not affected by any of the forementioned inhibitors. A specific inhibitor of this latter activity was not found.
4. The calculated activation energy of the high-affinity Ca^{2+}-ATPase calculated on the basis of specific activities at 25° and 37° was 57.9 kJ/mol.
5. In our membrane preparations the specific activities of high-affinity Ca^{2+}-ATPase and Na^+/K^+-ATPase were in the ratio of 1 to 20.4.
6. From these results we concluded that:
   a) eel gill branchial epithelial plasma membranes contain a high-affinity Ca^{2+}-ATPase with characteristics of a transport Ca^{2+}-ATPase;
   b) this activity is calmodulin dependent as it was inhibited by calmodulin antagonists and decreased by EGTA-treatment;

c) association of the high-affinity Ca^{2+}-ATPase activity and Na^+/K^+-ATPase activity in the same membrane fraction suggests that the Ca^{2+}-transport mechanism for transepithelial Ca^{2+}-transport is concentrated in the chloride cells of the gills.

INTRODUCTION

In terrestrial vertebrates intestinal absorption of Ca^{2+} performs a pivotal role in calcium metabolism. Studies with mammals and birds showed that intestinal absorption of Ca^{2+} is associated with an active transport process, which is regulated by 1α,25-dihydroxy-vitamin-D_3 (Van Os & Ghijsen, 1982; Wasserman *et al.*, 1982). Martin *et al.* (1969) were the first to report on the presence of a "vitamin-D" stimulated Ca^{2+}-ATPase activity in the brushborder of rat small intestine and comparable activities have subsequently been reported in whole homogenates and brushborder membranes (Kowarski & Schachter, 1973; Lane & Lawson, 1978) and basolateral membranes (Mircheff *et al.*, 1977) of mucosa of the small intestine.
However, this Ca^{2+}-ATPase activity was assayed at non-physiological Ca^{2+}-concentrations, had several characteristics which are different from the Ca^{2+}-transporting Ca^{2+}-ATPase of erythrocyte membranes, and as a result it was identified as a plasma membrane (alkaline) phosphatase (Holdsworth, 1970; Haussler *et al.*, 1970) rather than a classical Ca^{2+}-transporting enzyme. On the other hand it is dependent on vitamin-D and its activity is positively correlated with trans-epithelial Ca^{2+}-transport (Mircheff *et al.*, 1977), so that some involvement, not yet defined, in the process of calcium transport has been presumed.

Recently, Ghijsen & Van Os (1979) and Ghijsen *et al.* (1980) successfully demonstrated the presence of both an alkaline phosphatase and a transport Ca^{2+}-ATPase enzymic activity in the basolateral plasma membranes of rat enterocytes. Differentiation between these two enzyme activities was realized on the basis of inhibitor and substrate specificities. Moreover, studies on phosphorylated intermediates of the phosphatases of these membranes (De Jonge *et al.*, 1981) and on Ca^{2+}-transport in basolateral membrane vesicles (Ghijsen *et al.*, 1982) provided substantial evidence that Ca^{2+}-transport by the enterocyte plasma membrane must be attributed to a high-affinity Ca^{2+}-ATPase activity. This last enzyme, when assayed in the presence of 2 mM free Mg^{2+}, shows high affinity for Ca^{2+} ($K_{0.5}$ < 1 μM), prefers (Mg^{2+}-)ATP as a substrate

and is stimulated by calmodulin, three characteristics that are also associated with mammalian erythrocyte transport Ca^{2+}-ATPase (Vincenzi & Larsen, 1980). A similar enzyme has been demonstrated in avian oviduct shell gland (Coty & McConkey, 1982). Such Ca^{2+}-transport ATPases are now postulated to be generally present in all animal cells for the purpose of Ca^{2+}-extrusion to maintain low free Ca^{2+}-levels in the cytosol (Schatzmann, 1982). If this is correct it follows that high levels of this enzyme should be found in epithelia specialized for Ca^{2+}-transport such as the teleost gills but to our knowledge no pertinent reports have been published on these animals.

In this paper results are presented that demonstrate the presence of a high-affinity Ca^{2+}-ATPase in the gills of a teleost fish. Fresh water teleosts take up Ca^{2+} from the water almost exclusively via the gills (Berg, 1981), a process that can be stimulated by the hormone prolactin (Wendelaar Bonga & Flik, 1982). The molecular mechanisms by which prolactin exerts its effect on Ca^{2+}-uptake via the branchial epithelium are unknown. For many teleostean species branchial Ca^{2+}-ATPase activities have been reported (Ma *et al.*, 1974; Fenwick, 1978; Moon, 1978; Ho & Chan, 1980; Shephard, 1981; Doneen, 1981). Moreover, these activities are positively correlated with trans-epithelial transport rates of $^{45}Ca^{2+}$ in isolated gills (So & Fenwick, 1977). Recently we have shown that the characteristics of the putative branchial Ca^{2+}-ATPase activities, such as low apparent Ca^{2+}-affinity and alkaline pH optima, do not fulfil the requirements for a transport Ca^{2+}-ATPase; rather the characteristics of this branchial Ca^{2+}-ATPase are consistent with those of an alkaline phosphatase (Flik *et al.*, 1983). The observed Ca^{2+}-activated ATP-hydrolysis by these gill membrane phosphatases are most easily explained on the basis of chelation of Ca^{2+} with the substrate and not from stimulatory effects of Ca^{2+} on enzyme activities. We concluded that the assay conditions reported in the literature to measure gill Ca^{2+}-ATPase activities do not allow the resolution of a homogeneous transport Ca^{2+}-ATPase.

We now report studies on the substrate specificity of Ca^{2+}-induced ATP-hydrolysis in gill plasma membranes of the eel. Ca^{2+}-induced ATP-hydrolysis was assayed in the presence of 2 mM free Mg^{2+}, as recommended by Ghijsen & Van Os (1979) and Ghijsen *et al.* (1980; 1982), under conditions that allow predictions of free Ca^{2+} levels in the micromolar range. By so doing, we demonstrate that the high- rather than the low-affinity Ca^{2+}-ATPase in these membranes has characteristics common to those found in mammalian intestinal and erythrocyte Ca^{2+} transport systems.

Materials

All assays were performed in disposable phosphate-free plastic tubes. All reagents used were of the highest purity commercially available. Ultra pure water was used in all assays. Calmidazolium (R24571) was purchased from Janssen Pharmaceutica, Beerse, Belgium. All other chemicals were obtained from Sigma (St. Louis, Mo.).

Membrane isolation

Female yellow eels, *Anguilla rostrata* LeSueur, with an average body weight of 1.7 Kg were purchased in May 1982 from a commercial fish dealer in Québec City, Québec, Canada. The eels were held in running de-chlorinated Ottawa city tapwater (0.45 mM Ca^{2+}, 12°C) and under a photoperiod of 16 h of light alternating with 8 h of darkness until July and August 1982 when the experiments were performed. The animals were not fed.

After quick anesthesia in a Tris-buffered (pH 7.4) MS-222 solution (6 $g.L^{-1}$) the heart was exposed. A cannula was inserted into the bulbus arteriosus and the branchial apparatus was perfused with 30 ml of ice cold isotonic, heparin (20 U/ml) containing saline, until the gills were cleared of blood cells. The protease inhibitor PMSF (0.2 mM) was added to the perfusion fluid to enhance enzyme recovery. The branchial epithelium (about 2.5 g w.wt/Kg fish) was scraped off with a glass microscope slide onto an ice cold glass plate. All subsequent steps were performed at 0-4°C. A highly enriched plasma membrane fraction as judged from high Na^+/K^+-ATPase purification factors, with only minor mitochondrial contamination as judged from low recovery of succinic dehydrogenase activity, was obtained with centrifugation techniques (Flik *et al.*, 1983). Scrapings were disrupted (20 strokes) in large volumes (75 ml) of a hypotonic buffer with a loose-fitting Dounce homogenizer. The homogenate was centrifuged at 550 g for 15 min (Sorval RC-2B) to remove nuclei and cellular debris (pellet). The membranes and mitochondria were pelleted by centrifugation of the supernatant for 30 min at 27 K rpm (Beckman SW 28 rotor) and the pellet was resuspended with the same Dounce homogenizer (100 strokes) in an isotonic (sucrose) buffer containing EDTA (0.5 mM) to assure a leaky membrane preparation. The resulting membrane suspension was centrifuged differentially: 1 K g. 10 min, 10 K g. 10 min and 30 K g. 30 min (Sorval RC-2B). The final pellet was rinsed twice and subsequently resuspended in a buffer containing

20 mM Hepes/Tris (pH 7.4), 100 mM NaCl and 5 mM $MgCl_2$. Aliquots of this suspension were rapidly frozen in liquid N_2 and stored at -90°C for a maximum of 3 weeks. Recovery in the gill membrane preparation with respect to the crude homogenate, was: 2.6 ± 0.57% (n=7) for the total protein, and 13.1 ± 2.33% for the total Na^+/K^+-ATPase. The Na^+/K^+-ATPase was concentrated 60-fold and only 3.4 ± 0.4% (n=7) of the initial succinic dehydrogenase activity remained in this preparation. To inhibit ATPase activities resulting from mitochondrial contamination, oligomycin B and sodium azide were added routinely to all assays for Ca^{2+}-stimulated ATPase activities. Detergent treatment (Tween-80 or Triton X-100, 0.1% v/v) did not affect Na^+/K^+-ATPase activities of membrance preparations isolated in the presence of EDTA. A maximum increase of 120% in Na^+/K^+-ATPase specific activities was observed after detergent treatment of membranes isolated in the absence of EDTA. These observations were taken as evidence that the use of EDTA during isolation produced the leaky vesicle preparation necessary for optimal substrate and ion accessibility. The Ca^{2+}-concentration of this membrane suspension was 100 nmol Ca^{2+} per mg BSA equivalents membrane protein.

Enzyme assays

Ca^{2+}-stimulated ATPase activities were assayed as described by Ghijsen *et al.* (1980; 1982), in a medium containing 100 mM NaCl, 5 mM $MgCl_2$, 3 mM Na_2ATP, 5 mM NaN_3, 0.1 mM ouabain, 5 µg/ml oligomycin B and 20 mM Hepes/Tris (pH 7.4). A 1 mM capacity Ca^{2+}-buffer system (0.5 mM EGTA + 0.5 mM NTA) was used to ensure predictable free Ca^{2+} levels in the micromolar range. Ca^{2+}-induced ATP-hydrolysis was taken as the difference in P_i-release in the presence and absence of Ca^{2+}. Membrane suspensions (20 µl, 14.1 ± 0.5 µg BSA equivalents) were mixed on ice with 500 µl ice cold assay medium and the enzyme reactions were started by incubation in a shaking water-bath at 25°C or 37°C for 30-45 min. The reaction was stopped on ice with 100 µl ice cold TCA (40% w/v) and phosphate was analyzed as described by Fenwick (1976). Stimulation of ADP- and p-NPP-hydrolysis by Ca^{2+} was determined in the same medium but ATP was replaced by 3 mM of the different substrates. In these cases the free Ca^{2+}-concentrations were calculated, taking into account the respective stability constants of the substrates and Ca^{2+}. Chlorpromazine, trifluoperazine and calmidazolium stock solutions were prepared in ethanol on the day of assay. To prevent formation of free radicals trifluoperazine solutions were protected from light.

Effects of phenothiazins and calmidazolium were tested at constant membrane protein concentrations to exclude variation in inhibitor concentrations due to the lipophilic interactions with membranes suggested by Hinds *et al.* (1981).

Protein, Na^+/K^+-ATPase, p-NPPase and succinic dehydrogenase were assayed as described by Flik *et al.* (1983). The concentrations of stock Ca^{2+}-solutions were checked by atomic absorption spectrophotometry in the presence of La^{3+} (20 mM). Statistical analysis of the data was carried out with Student's t-test. Significance was accepted with $p < 0.05$ when $n \leq 4$ or with $p < 0.02$ when $n > 4$ (two-sided, α=5%). Results are expressed as mean values ± standard error of the mean (S.E.M.). Linear regression analysis was based on the least-squares method. Kinetic parameters of Ca^{2+}-stimulated phosphatase activities were calculated after fitting the data with an iterative procedure based on the least-squares method using a computer programme developed for a two-site single carrier transport model (Borst Pauwels, 1973).

RESULTS

Ca^{2+}-stimulation and substrate specificity

In Fig. 1 the results of substrate preference tests are shown for Ca^{2+}-stimulated phosphatase activity in eel gill plasma membranes. At Ca^{2+}-concentrations lower than 100 µM, in the presence of 2 mM free Mg^{2+}, stimulations of ATP-hydrolysis is significantly greater than hydrolysis of ADP ($P < 0.001$).

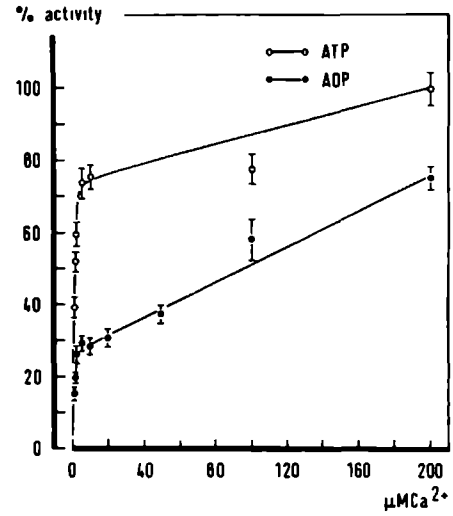

Figure 1. Effect of Ca^{2+} on ATP- and ADP-hydrolysis by plasma membrane of eel branchial epithelium. Activities are expressed as percentages of maximum ATP-hydrolysis obtained at 200 µM free Ca^{2+} (= 100%). Hydrolysis of ATP and ADP in the absence of Ca^{2+} were 41.01 ± 4.79 and 33.2 ± 5.48 µmol $P_i.h^{-1}$.mg protein^{-1}, respectively; 200 µM free Ca^{2+}-induced hydrolysis of ATP and ADP were 8.94 µmol $P_i.h^{-1}$.mg protein^{-1} and 6.71 µmol $P_i.h^{-1}$.mg protein^{-1}, respectively. Mean values ± S.E.M.; n=6 in all cases. o ——— o ATP, • ——— • ADP.

At higher Ca^{2+}-concentrations no significant difference was observed between Ca^{2+}-induced ATP- and Ca^{2+}-induced ADP-hydrolysis ($P > 0.15$). Eadie-Hofstee plots (Fig. 2) of Ca^{2+}-induced ATP- and Ca^{2+}-induced ADP-hydrolysis reveal that two Ca^{2+} affinity sites are present when either substrate was used. Computed K_m-values for the high-affinity sites amount to 0.22 µM Ca^{2+} for ATP-hydrolysis and 0.32 µM Ca^{2+} for ADP-hydrolysis, whereas the low-affinity sites have K_m values of 0.535 mM Ca^{2+} for ATP-hydrolysis and 0.230 mM Ca^{2+} for ADP-hydrolysis (Table I). Clearly these results indicate the simultaneous presence of high-affinity Ca^{2+}-ATPase and low-affinity phosphatase activities in plasma membranes of eel branchial epithelium.

Effects of phenothiazins and calmidazolium on Ca^{2+}-induced ATP hydrolysis

To further differentiate between either activity we investigated the effects of well-known inhibitors of high-affinity transport Ca^{2+}-ATPase and non-specific phosphatases on Ca^{2+}-induced substrate hydrolysis in the presence of 1 or 200 µM free Ca^{2+} as suggested by Ghijsen *et al.* (1980). As shown in Table II, chlorpromazine (10^{-5} M), trifluoperazine (10^{-5} M) and calmidazolium (10^{-6} M) inhibited 1 µM Ca^{2+}-induced ATP hydrolysis. Calmidazolium gave complete inhibition without significantly affecting background activities (= zero Ca^{2+}). Trifluoperazine was a more powerful inhibitor than chlorpromazine, but in contrast to the latter inhibitor it also significantly affected background activities. All three inhibitors, at the low concentrations tested, are thought to display their effects as calmodulin antagonists, with calmidazolium being the most potent and specific substance (Van Belle, 1981). We therefore tested the effects of EGTA-treatment and calmidazolium on 1 µM Ca^{2+}-induced ATP-hydrolysis. EGTA-treatment decreased the Ca^{2+}-ATPase activity more than three-fold and led to 35% decrease in background activities.

Figure 2. Eadie-Hofstee plots of Ca^{2+}-induced ATP- and ADP-hydrolytic activities in plasma membranes of eel branchial epithelium at various calcium concentrations. Enzymic activities (V) are expressed as percentage stimulation above background hydrolytic activity. Ca^{2+}-concentrations (S) are expressed in µM. Background activities are 41.01 ± 4.79 and 33.2 ± 5.48 µmol $P_i.h^{-1}.mg\ protein^{-1}$ for ATP and ADP, respectively. Computed $K_{0.5}$ and V_{max} values are presented in Table I. o ——— o ATP, ● ——— ● ADP. Results are presented as mean values of at least 6 determinations.

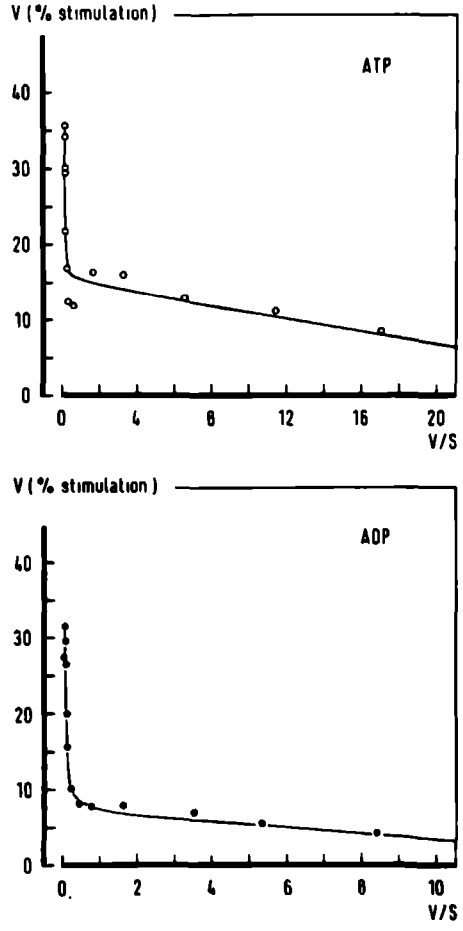

Table I. Kinetic parameters of Ca^{2+}-stimulated ATPase and ADPase activities in plasma membranes of eel branchial epithelium.

$K_{0.5}$-values are expressed in µM Ca^{2+} and V_{max}-values in µmol $P_i.h^{-1}.mg\ protein^{-1}$. Enzyme activities were assayed in the presence of up to 5 mM free Ca^{2+} and 2 mM free Mg^{2+} and either 3 mM ATP or 3 mM ADP. V_{max}-values of the low-affinity sites were corrected for substrate hydrolysis resulting from stimulation of the high-affinity sites. Values were computed from Eadie-Hofstee plots after fitting the data on the basis of a general rate equation for a two-site transport model by means of an iterative procedure (Borst Pauwels, 1973). Mean values ± S.E.M. are given; n=6.

	High-affinity site		Low-affinity site	
Substrate	$K_{0.5}$	V_{max}	$K_{0.5}$	V_{max}
ATP	0.22	5.41 ± 0.63	535	10.70 ± 1.25
ADP	0.32	2.32 ± 0.38	230	8.50 ± 1.40

Table II. Effects of chlorpromazine, trifluoperazine, calmidazolium and EGTA-treatment on background ATPase and Ca^{2+}-ATPase activities in plasma membranes of eel branchial epithelium.

Enzyme activities are expressed as µmol $P_i.h^{-1}.mg\ protein^{-1}$ and were assayed at 37°C. Inhibitors were dissolved in ethanol. Final ethanol concentrations in the assay media did not exceed 0.1% v/v ethanol (= controls). Membrane suspensions were pre-incubated with inhibitors or solvent for 15 min at 37°C as suggested for calmidazolium (Van Belle, 1981). EGTA-treatment consisted of resuspension of membranes with a loose-fitting Dounce homogenizer (100 strokes) in 10 volumes of 20 mM Hepes/Tris (pH 6.8), 100 mM NaCl and 5 mM EGTA; membranes were collected by centrifugation and subsequently washed two times with the standard basis assay buffer (see Materials and Methods). Mean values ± S.E.M. are given with the number of observations in parentheses.

Treatment	Enzyme activities: Background ATPase	Ca^{2+}-ATPase
Controls	45.7 ± 3.6 (26)	3.45 ± 0.35 (26)
Chlorpromazine (10^{-5} M)	41.9 ± 3.2 (26)	0.43 ± 0.03 (26)
Trifluoperazine (10^{-5} M)	34.7 ± 2.7 (26)**	0.14 ± 0.10 (26)
Calmidazolium (10^{-6} M)	40.3 ± 3.0 (26)	N.S.* (26)
EGTA + Ethanol 0.1% v/v	29.8 ± 4.7 (8)**	1.05 ± 0.04 (8)
EGTA + Calmidazolium (10^{-6} M)	32.1 ± 4.4 (8)	N.S.*

* No significant stimulation above background ATP hydrolysis in the absence of Ca^{2+}.
** Significantly different from control values $p < 0.001$.

Table III. Effects of calmidazolium on 200 µM Ca^{2+}-induced ATP- or ADP-hydrolysis.

Enzyme activities are expressed as µmol $P_i.h^{-1}$.mg $protein^{-1}$ and were assayed at 37°C. Membrane suspensions were pre-incubated as described in Table II. Background ATP- and ADP-hydrolytic activities (zero Ca^{2+}) were not affected by calmidazolium (10^{-6} M). Background activities were 38.5 ± 2.02 and 35.8 ± 1.92 µmol $P_i.h^{-1}.protein^{-1}$ for controls and calmidazolium incubated samples with ATP, respectively; and 25.5 ± 2.56 and 25.33 ± 4.69 µmol $P_i.h^{-1}$.mg $protein^{-1}$ for controls and calmidazolium incubated samples with ADP, respectively. Values are means ± S.E.M. (n=6).

Substrate	200 µM Ca^{2+}-induced substrate hydrolysis (µmol $P_i.h^{-1}$.mg $protein^{-1}$): Controls (ethanol 0.1% v/v)	Calmidazolium (10^{-6} M)
ATP	4.06 ± 2.49	3.86 ± 0.97
ADP	4.22 ± 1.29	4.25 ± 0.49

The Ca^{2+}-ATPase activity resulting after EGTA-treatment could be further specifically inhibited by calmidazolium, without significantly affecting background activities. Table III shows the effects of calmidazolium on 200 µM Ca^{2+}-induced hydrolysis of ATP and ADP. Neither activity was significantly affected. These observations suggest a specific inhibition of the high-affinity Ca^{2+}-ATPase activity by calmidazolium, whereas low-affinity phosphatase activities were not affected.

Effects of theophylline on background and Ca^{2+}-stimulated ATP hydrolysis

As can be seen in Table IV, background ATPase activities at low free Mg^{2+} concentrations (3 mM ATP + 3 mM Mg^{2+}) are theophylline-sensitive, maximum inhibition amounts to 17.7 ± 2.2% at 1.25 mM theophylline. This observation suggests the occurrence of a non-specific alkaline phosphatase activity (Fawaz & Tejirian, 1972). In the presence of 2 mM free Mg^{2+} background ATPase activities decreased and no theophylline inhibition occurred. When p-NPP was substituted for ATP no significant effects of theophylline were observed either at 3 or 5 mM Mg^{2+}. Specific activities were low for p-NPP when compared to ATP as substrate; specific activities were significantly lower at 5 mM than at 3 mM Mg^{2+} ($P < 0.05$). Further, theophylline did not inhibit either 1 µM Ca^{2+} or 200 µM Ca^{2+}-induced ATP-hydrolysis at 2 mM free Mg^{2+} (Table V).

Table IV. Effect of theophylline on background hydrolysis of ATP and p-NPP in plasma membranes of eel branchial epithelium.

Enzyme activities are expressed as µmol $P_i.h^{-1}.mg\ protein^{-1}$ and were assayed at 37°C. Mean values ± S.E.M. are given with the number of experiments in parentheses.

Substrate	Controls	1.25 mM theophylline	Inhibition
3 mM ATP + 3 mM Mg^{2+}	45.2 ± 3.5 (6)	37.2 ± 4.1 (6)	17.7 ± 2.2*
3 mM p-NPP + 3 mM Mg^{2+}	4.01 ± 1.17 (4)	3.21 ± 0.79 (4)	N.S.
3 mM ATP + 5 mM Mg^{2+}	39.1 ± 4.5 (6)	39.9 ± 5.2 (6)	N.S.
3 mM p-NPP + 5 mM Mg^{2+}	2.53 ± 0.17 (3)	2.48 ± 0.53 (3)	N.S.

*$P < 0.01$

Table V. Effect of theophylline on Ca^{2+}-induced ATP-hydrolysis in plasma membranes of eel branchial epithelium.

Enzyme activities are expressed as µmol $P_i.h^{-1}.mg\ protein^{-1}$ and were assayed at 37°C in the presence or absence of 2.0 mM theophylline. Background ATPase activities were not significantly affected by theophylline. Mean values ± S.E.M. are given with the number of experiments in parentheses.

	1 μM free Ca^{2+}	200 μM free Ca^{2+}
Control	3.13 ± 0.72 (11)	4.40 ± 0.47 (3)
2.0 mM theophylline	3.18 ± 0.40 (14)	4.40 ± 0.53 (3)

Effects of temperature on Ca^{2+}-induced ATP-hydrolysis

Table VI gives the specific activities of 1 μM Ca^{2+}-induced ATP-hydrolysis assayed at 25°C and 37°C. Assuming linearity of the temperature dependency of this activity in this temperature range, we calculated the activation energy from an Arrhenius plot. A value of 13.84 Kcal/mol was found.

Table VI. Effect of temperature on Ca^{2+}-induced ATP-hydrolysis in plasma membranes of eel branchial epithelium.

Enzyme activities are expressed as μmol $P_i.h^{-1}.mg\ protein^{-1}$ and were assayed in the presence of 1 μM Ca^{2+}. Association constants of NTA and EGTA for Ca^{2+}, used to calcule 1 μM free Ca^{2+}, are corrected for temperature according to Scharff (1979). Activation energy is calculated from the slope of the ln(activity) vs. T^{-1}-curve. Mean values ± S.E.M. are given (n=6).

Temperature	Ca^{2+}-induced ATP-hydrolysis (μmol $P_i.h^{-1}.mg\ protein^{-1}$)
25°C	1.81 ± 0.24 (6)
37°C	4.46 ± 0.61 (6)

Activation energy: 57.9 $kJ.mol^{-1}$

Comparison of ATPase activities in gill plasma membranes

In Table VII high-affinity Ca^{2+}-ATPase specific activities are compares with specific activities of Na^+/K^+-ATPase and (Ca^{2+}∿ATP)phosphatase (P_i-release in the presence of 2 mM Ca^{2+} and 5 mM ATP; Flik *et al.*, 1983). Values for Na^+/K^+-ATPase were about 20 times higher than those for Ca^{2+}-ATPase, whereas (Ca^{2+}∿ATP)phosphatase activities surpassed Ca^{2+}-ATPase activities more than 30-fold.

Table VII. ATP-hydrolytic activities in plasma membranes of eel branchial epithelium.

Enzyme activities are expressed as µmol $P_i.h^{-1}.mg\ protein^{-1}$ and were assayed at 37°C. For Na^+/K^+-ATPase and Ca∿ATP-phosphatase assay conditions is referred to Flik *et al.* (1983)and Fenwick(1978) respectively. High-affinity Ca^{2+}-ATPase is assayed at 1 µM free Ca^{2+} in the presence of 2 mM free Mg^{2+}. Mean values ± S.E.M. are given (n=26).

	Specific activity (µmol $P_i.h^{-1}.mg\ protein^{-1}$)
Na^+/K^+-ATPase	71.3 ± 6.7
Ca^{2+}-ATPase	3.49 ± 0.35
Ca^{2+}∿ATP-phosphatase	107.6 ± 5.1

DISCUSSION

The results presented in this paper show for the first time the presence of a true high-affinity Ca^{2+}-ATPase in the plasma membranes of the branchial epithelium of bony fishes. Further, the characteristics of this Ca^{2+}-ATPase resemble those of the transport Ca^{2+}-ATPase found in the intestine (Ghijsen *et al.*, 1980), kidney (Van Os & Ghijsen, 1981) and erythrocytes (Gietzen *et al.*, 1981) of mammals and oviduct shell gland of birds (Coty & McConkey, 1982). The following observations lead us to consider the high-affinity Ca^{2+}-ATPase activity described here as the plasma membrane transport Ca^{2+}-ATPase:

1) the high Ca^{2+}-affinity (K_{Ca} : 0.22 µM) observed in the presence of excess free Mg^{2+} indicates that this activity can be specifically stimulated by Ca^{2+} at intracellular concentrations;
2) phosphatase activity with such high affinity for Ca^{2+} preferentially hydrolyzes ATP;
3) the observed high-affinity Ca^{2+}-ATPase activity was inhibited by calmodulin antagonists thus suggesting its calmodulin dependency;
4) the high-affinity Ca^{2+}-ATPase activity was not affected by theophylline or L-phenylalanine which are inhibitors of plasma membrane Ca^{2+}-stimulated alkaline phosphatase activities (Ghijsen *et al.*, 1980);
5) the specific activity of the branchial high-affinity Ca^{2+}-ATPase (V_{max} : 5.41 ± 0.63 µmol $P_i.h^{-1}.mg\ protein^{-1}$) is consistent with those reported

for transport Ca^{2+}-ATPases of mammalian adipocyte plasma membranes (6.0 µmol $P_i.h^{-1}.mg\ protein^{-1}$; Pershadsingh *et al.*, 1980) and for erythrocyte plasma membranes (3.6 µmol $P_i.h^{-1}.mg\ protein^{-1}$; Waisman *et al.*, 1981);

6) the calculated activation energy for the branchial high-affinity Ca^{2+}-ATPase (13.48 $Kcal.mol^{-1}$) is close to the values reported for mammalian erythrocyte Ca^{2+}-ATPase (14-19 $Kcal.mol^{-1}$; Larsen *et al.*, 1978);

7) the branchial high-affinity Ca^{2+}-ATPase activity is indeed most likely to be located in the plasma membranes of the epithelium: it is closely associated with Na^+/K^+-ATPase activity, a basolateral plasma membrane marker (Mircheff & Wright, 1976) and it is insensitive to oligomycin B and sodium azide, which are inhibitors of mitochondrial ATPases (Katz & Doucet, 1980).

Previously we showed that the activity formerly described as Ca^{2+}-ATPase activity in eel gills in fact represents a heterogeneous pool of phosphatases that depend on Ca^{2+}-activation for ATP-hydrolysis (Flik *et al.*, 1983). As was pointed out by Ghijsen *et al.* (1980) for rat intestinal plasma membranes, a transport Ca^{2+}-ATPase enzymic activity should be defined as the ATPase activity that is stimulated by free Ca^{2+} at intracellular concentrations in the presence of a surplus Mg^{2+}. This can be established only under assay conditions with known free Ca^{2+}-levels such as in the presence of a Ca^{2+}-buffer system similar to the one used in this study. Previous studies on Ca^{2+}-ATPase activity in fish gills have not satisfied these requirements (Flik *et al.*, 1983).

The Ca^{2+}-induced ATP-hydrolysis by branchial plasma membrane phosphatase activity could be separated into two distinct kinetic components, one with high affinity and one with low affinity for Ca^{2+}. For the high-affinity component ($K_{0.5}$: 0.22 µM Ca^{2+}) ATP was preferred over ADP, the low-affinity component ($K_{0.5}$: 230-535 µM Ca^{2+}) showed no preference. We consider only the first component as an ATPase involved in Ca^{2+}-transport. Such an enzyme would have an intracellular Ca^{2+}binding site similar to the comparable enzyme in mammalian intestine and, therefore, a $K_{0.5}$-value in the range of intracellular Ca^{2+}-concentrations. In general, $K_{0.5}$-values smaller than 1 µM Ca^{2+} have now been accepted as an important characteristic of enzymes such as Ca^{2+}-ATPases, adenylate cyclase and cyclic-AMP-phosphodiesterase, that are activated intracellularly (Cheung, 1980). The high-affinity component described here fulfils this criterion. It should be noted that such a $K_{0.5}$-value for Ca^{2+} is determined by the stability constant of the ligands in the Ca^{2+}-buffer used to calculate the free Ca^{2+}-concentrations (Reed & Bygrave, 1975; Scharff, 1979). In our study a $K_{Ca\text{-}EGTA}$-value of $10^{10.90}$ and a $K_{Ca\text{-}NTA}$-value of $10^{6.40}$, as suggested by Ghijsen *et al.* (1982) were used.

These values were taken from the studies of Scharff, (1979) who reported closely correlated values for observed and calculated Ca^{2+}-concentrations. The presence of two saturable components for Ca^{2+}-stimulated ATPase activities in plasma membranes has been reported too for e.g. pancreatic islet cells (Pershadsingh *et al.*, 1980), rat enterocytes (Ghijsen & Van Os, 1979) and Ehrlich ascites tumor cells (Klaven *et al.*, 1983) and seems of wider occurrence.

In substrate specificity tests with p-NPP, AMP, ADP and ATP, only hydrolysis of the latter two substrates was significantly stimulated by Ca^{2+}. ATP and ADP apparently can serve as substrates for both kinetic components, although the high-affinity component prefers ATP over ADP. Half maximal activation of Ca^{2+}-induced ATP and ADP hydrolysis by the low-affinity component, however, was calculated to occur at significantly different concentrations (535 and 230 µM Ca^{2+}, respectively). The significant release of ADP that will result from the high background activity when ATP is used as a substrate (Flik *et al.*, 1983) may have led to competitive inhibition of ATP-hydrolysis and therefore the $K_{0.5}$-value for Ca^{2+} of ATP-hydrolysis may be overestimated. Nevertheless, even the low-affinity site $K_{0.5}$-value for ADP-hydrolysis (230 µM Ca^{2+}) is too high by at least one order of magnitude to characterize an enzyme functioning at intracellular Ca^{2+}-concentrations. It is unlikely then that this component represents the activity of a Ca^{2+}-transporting enzyme. One striking observation during the substrate specificity tests was that our preparation does not increase the hydrolysis of p-NPP or AMP following addition of Ca^{2+}. In this respect it is quite distinct from the preparation of rat enterocyte basolateral plasma membranes used by Ghijsen *et al.* (1980). These authors differentiated between transport Ca^{2+}-ATPase and Ca^{2+}-stimulated non-specific phosphatase activity in their preparation, inter alia on the basis of substrate specificity: the high-affinity Ca^{2+}-ATPase activity proved to be ATP-dependent, whereas their low-affinity phosphatase activity showed no distinct preference for either p-NPP, AMP, ADP or ATP.

The specific inhibitory action of calmodulin antagonists on the high-affinity Ca^{2+}-ATPase activity suggested the presence of calmodulin in our membrane preparation. Although we did not assay the calmodulin content of these membrane preparations, its presence was indicated by the following observations:

1) Our membrane preparation still contained Ca^{2+}, even though EDTA was used during some steps of the isolation procedure and it may thus be concluded that the endogenous calmodulins were not necessarily extracted from the membranes. Indeed Lynch & Cheung (1979) have shown that the human erythrocyte Ca^{2+}-ATPase-calmodulin complex dissociates only after all Ca^{2+} is extracted.

2) Very low $K_{0.5}$-values for Ca^{2+}, as found for our high-affinity Ca^{2+}-ATPase activity (0.22 µM), have been shown to be indicative of the presence of calmodulin in erythrocyte Ca^{2+}-ATPase preparations (Schatzmann, 1982).
3) High-affinity Ca^{2+}-ATPase activity in our preparation was decreased by 70% after EGTA-treatment of the membranes.
4) In comparable studies using membranes of tilapia gills we showed the presence of calmodulin by means of radioimmunoassay or the cyclic nucleotide phosphodiesterase assay (unpublished observations).

The effects of the powerful and specific inhibitor (Larsen *et al.*, 1978; Van Belle, 1981) calmidazolium on the Ca^{2+}-induced ATP-hydrolysis are consistent with the assumption that only the 1 µM Ca^{2+}-induced ATP-hydrolysis is caused by an intracellularly activated calmodulin-dependent enzyme: this high-affinity component was inhibited, whereas the 200 µM Ca^{2+}-induced ATP-hydrolysis was not affected. Yet, the observed effect of calmidazolium on the high-affinity Ca^{2+}-ATPase activity in EGTA-treated membranes points to the possibility that this inhibitor may also have had some additional non-specific effects at 10^{-6} M. We observed that calmidazolium in its capacity to inhibit the high-affinity Ca^{2+}-ATPase activity was at least 10 times more effective than the phenothiazins. Chlorpromazine and trifluoperazine, at a concentration of 10^{-5} M, both significantly inhibited the high-affinity Ca^{2+}-ATPase activity, but only trifluoperazine markedly affected background activities. This action of trifluoperazine suggested some non-specific action. In a recent paper, Ho *et al.* (1983) showed that trifluoperazine at relatively high concentrations (viz. 10^{-5} M) shifted the Ca^{2+}-dependency of sarcoplasmic reticulum Ca^{2+}-ATPase to higher Ca^{2+}-concentrations. They further showed that trifluoperazine's inhibitory action results not from an interaction with the enzyme, but rather from an effect on the membrane constitution resulting in structural perturbations of the ATPase. Keeffe *et al.* (1980) reported for rat liver plasma membranes that chlorpromazine alters membrane fluidity and inhibits ATPase activities non-competitively over a 2-200 µM concentration range of the inhibitor. Calmidazolium on the other hand has been reported to surpass the phenothiazins in potency and to exert its effects at low inhibitor concentrations, and independently of Ca^{2+}, as a purely competitive calmodulin antagonist (Gietzen *et al.*, 1981). As 10^{-6} M calmidazolium inhibited high-affinity but not low-affinity Ca^{2+}-ATPase activity, we tentatively conclude that the latter activity represents calmodulin-independent phosphatase activity. Thus, this characteristic further differentiates

between phosphatase activity and high-affinity Ca^{2+}-ATPase activity.

Ca^{2+}-induced ATP-hydrolysis in rat enterocyte plasma membranes may partly result from alkaline phosphatase activity and this activity can be inhibited by L-phenylalanine and theophylline (Ghijsen *et al.*, 1980), well-known inhibitors of alkaline phosphatase activity (Fawaz & Tejirian, 1972; Ghosh & Fishman, 1966). We tested both inhibitors but under the present incubation conditions we observed no effect on Ca^{2+}-stimulated ATP-hydrolysis in gill plasma membranes. However, when free Mg^{2+}-levels were low we did observe theophylline inhibition of background ATPase activity, thus corroborating an earlier observation (Flik *et al.*, 1983). At 2 mM free Mg^{2+} (estimated) background activities decreased about 15% and theophylline sensitivity was not found. Background hydrolysis of p-NPP was very low and was not theophylline sensitive at either 3 or 5 mM Mg^{2+}. The weak hydrolysis of p-NPP seemed to indicate that the non-specific phosphatase activity of eel gills is distinct from alkaline phosphatases found in teleost intestine (Flik *et al.*, 1982) or mammalian liver and intestine (Jensen, 1979). To date we have not identified an effective inhibitor which can specifically block the ATP-hydrolysis resulting from the Ca^{2+}-stimulated low-affinity component.

In mammals, enzymatic and ion transport studies showed that the transport Ca^{2+}-ATPase and Na^+/K^+-ATPase occur in the same tissue plasma membranes (Mircheff *et al.*, 1983; Ghijsen *et al.*, 1982). Similarly, both enzymes were found together in the same membranes from eel gills. Transepithelial Na^+-transport in the gills is generally believed to be mediated by the chloride cells of the branchial epithelium (Maetz & Bornancin, 1975). Indeed Hootman & Philpott (1979) have provided ultracytochemical evidence that the bulk of the fish gill Na^+/K^+-ATPase activity is located within the reticular system of the chloride cells. As in the present study high-affinity Ca^{2+}-ATPase and Na^+/K^+-ATPase activities were found in close association in gill cell membranes we suggest that the Ca^{2+}-transport ATPase is also mainly located in the reticular system (an extension of the plasma membrane) of the chloride cells. In fresh water teleosts branchial Ca^{2+} uptake occurs against a transepithelial Ca^{2+}-gradient. The branchial epithelium is a typical tight epithelium and therefore it is likely that transport of Ca^{2+} in the gills follows a transcellular route through the chloride cells. This transport will involve similar events as transcellular Ca^{2+}-transport in e.g. mammalian enterocytes:

1) entry of Ca^{2+} down its electrochemical gradient at the apical membrane,
2) passage through the cell, mediated by cytosolic Ca^{2+}-binding protein, and
3) extrusion of Ca^{2+} to the extracullular fluid by a transport Ca^{2+}-ATPase located in the so-called reticular system. The present observations suggest that Ca^{2+}-

transport in the gills, at least to a large extent, is a function of the chloride cells of this epithelium.

The ratio of the specific activities of Na^+/K^+-ATPase and high-affinity Ca^{2+}-ATPase in our membrane preparation (71.3/3.49=20.4) is very similar to values reported by De Jonge *et al.* (1981) for such activities in rat enterocyte basolateral plasma membranes (33.9/2.1=16.4). This similarity points to an analogous mechanism for the transcellular transport of Ca^{2+}- and Na^+ ions in teleostean gills and mammalian intestinal epithelium.

We are still left with the problem of explaining the function of the branchial alkaline phosphatase (low-affinity "Ca^{2+}-ATPase" of the fish literature). The activity of this enzyme appears to reflect the rate of transepithelial calcium transport in eel gills (So & Fenwick, 1977). Alkaline phosphatase has been implicated in calcium transport in rat intestine (Halloran & DeLuca, 1981). Further time course studies of hormonal effects on this branchial enzyme are under way.

REFERENCES

BERG, A. (1981) Studies on the metabolism of calcium and strontium in freshwater fish. I. Relative contribution of direct and intestinal absorption. Me.Inst.Ital.Idrobiol. 23: 161-196.

BORST PAUWELS, G.W.F.H. (1973) Two site - single carrier transport kinetics. J.theor.Biol. 40: 19-31.

CHEUNG, W.Y. (1980) Calmodulin plays a pivotal role in cellular regulation. Science 207: 19-27.

COTY, W.A. & McCONKEY Jr C.L. (1982) A high-affinity calcium stimulated ATPase activity in the hen oviduct shell gland. Arch.Biochem.Biophys. 219: 444-453.

DONEEN, B.A. (1981) Effects of adaptation to seawater, 170% seawater, and to freshwater on activities and subcellular distribution of branchial Na^+-K^+-ATPase, low- and high affinity Ca^{2+}-ATPase and ouabain insensitive ATPase in *Gillichthys mirabilis*. J.Comp.Physiol. 145: 51-61.

DE JONGE, H.R., GHIJSEN, W.E.J.M. & VAN OS, C.H. (1981) Phosphorylated intermediates of Ca^{2+}-ATPase and alkaline phosphatase in plasma membranes from rat duodenal epithelium. Biochim.Biophys.Acta 547: 140-149.

DE SMEDT, H., PARYS, J.B., BORGHGRAEF, R. & WUYTACK, F. (1983) Phosphorylated intermediates of (Ca^{2+} + Mg^{2+})-ATPase and alkaline phosphatase in renal plasma membranes. Biochim.Biophys.Acta 728: 409-418.

FAWAZ, E.N. & TEJIRIAN, A. (1972) Inhibition of alkaline phosphatase by theophylline *in vitro*. Hoppe-Seyler's Z.Physiol.Chem. 353: 1779-1783.

FENWICK, J.C. (1976) Effect of stanniectomy on calcium-activated adenosine triphosphatase activity in the gills of freshwater adapted North American eels, *Anguilla rostrata* LeSueur. Gen.Comp.Endocrinol. 29: 283-287.

FENWICK, J.C. (1978) Ca^{2+}-activated adenosinetriphosphatase activity in the gills of freshwater- and seawater-adapted eels *(Anguilla rostrata)*. Comp.Biochem. Physiol. 62B: 67-70.

FLIK, G., REIJNTJENS, F.M.J., STIKKELBROECK, J. & FENWICK, J.C. (1982) 1,25-Vitamin D_3 and calcium transport in the gut of tilapia *(Sarotherodon mossambicus)*. J.Endocrinol. 94: 40P.

FLIK, G., WENDELAAR BONGA, S.E. & FENWICK, J.C. (1983) Ca^{2+}-dependent phosphatase and ATPase activities in plasma membranes of the gill epithelium. I. Identification of Ca^{2+}-activated ATPase activity with non-specific alkaline phosphatase activity. Comp.Biochem.Physiol. 67B: 745-754.

GHOSH, N. & FISHMAN, W.H. (1966) On the mechanism of inhibition of intestinal alkaline phosphatase by L-phenylalanine. G.Biol.Chem. 241: 2516-2522.

GHIJSEN, W.E.J.M. & VAN OS, C.H. (1979) Ca^{2+}-stimulated ATPase in brush border and basolateral membranes of rat duodenum with high-affinity sites for Ca ions. Nature 279: 802-803.

GHIJSEN, W.E.J.M., DE JONG, H.R. & VAN OS, C.H. (1980) Dissociation between Ca^{2+}-ATPase and alkaline phosphatase activities in plasma membranes of rat duodenum. Biochim.Biophys.Acta 599: 538-551.

GHIJSEN, W.E.J.M., DE JONG, M.D. & VAN OS, C.H. (1982) ATP-dependent calcium transport and its correlation with Ca^{2+}-ATPase activity in basolateral plasma membranes of rat duodenum. Biochm.Biophys.Acta 689: 327-336.

GIETZEN, K., WÜTHRICH, A. & BADER, H. (1981) R24571: a new powerful inhibitor of red blood cell Ca^{2+}-transport ATPase and of calmodulin-regulated functions. Biochem.Biophys.Res.Commun. 101: 418-425.

GIETZEN, K., KONRAD, R., TEJČKA, M., FLEISCHER, S. & WOLF, H.U. (1981) Purification, characterization and reconstitution of the Ca^{2+}-transport system (high-affinity Ca^{2+}, Mg^{2+}-ATPase) of the human erythrocyte membrane. Acta Biol.Med.Germ. 40: 443-456.

HALLORAN, B.P. & DeLUCA, H.F. (1981) Intestinal calcium transport: evidence for two distinct mechanisms of action of 1,25-dihydroxy vitamin D_3. Arch.Biochem. Biophys. 208: 477-486.

HAUSSLER, M., NAGODE, L. & RASMUSSEN, H. (1970) Induction of intestinal brush border alkaline phosphatase by vitamin D and its identity with Ca-ATPase. Nature 228: 119-120.

HINDS, T.R., RAESS, B.U. & VINCENZI, F.F. (1981) Plasma membrane Ca^{2+}-transport: antagonism by several potential inhibitors. J.Membrane Biol. 58: 57-65.

HO, S.M. & CHAN, D.K.O. (1980) Branchial ATPases and ionic transport in the eel *Anguilla japonica*. II. Ca^{2+}-ATPase. Comp.Biochem.Physiol. 67B: 639-645.

HO, M.M., SCALES, D.J. & INESI, G (1983) The effect of trifluoperazine on the sarcoplasmic reticulum membrane. Biochim.Biophys.Acta 730: 64-70

HOLDSWORTH, E.S. (1970) The effect of vitamin D on enzyme activities in the mucosal cells of the chick small intestine. J.Membrane Biol. 3: 43-53.

HOOTMAN, S.R. & PHILPOTT, C.W. (1979) Ultracytochemical localization of Na^+, K^+-activated ATPase in chloride cells from the gills of a euryhaline teleost. Anat.Rec. 193: 99-130.

JENSEN, M.H. (1979) Dephosphorylation of purine mononucleotides by alkaline phosphatases: substrate specificity and inhibition patterns. Biochim.Biophys.Acta 571: 55-62

KATZ, A.I. & DOUCET, A. (1980) Calcium activated adenosine triphosphatase along the rabbit nephron. Int.J.Biochem. 12: 125-129.

KEEFFE, E.B., BLANKENSHIP, N.M. & SCHARSCHMIDT, B.G. (1980) Alteration of rat liver plasma membrane fluidity and ATPase activity by chlorpromazine hydrochloride and its metabolites. Gastroenterol. 79: 222-231.

KLAVEN, N.V., PERSHADSINGH, H.A., HENIUS, G.V., LARIS, P.C., LONG, J.W.Jr., & McDONALD, J.M. (1983) A high-affinity, calmodulin-sensitive (Ca^{2+}+Mg^{2+})-ATPase and associated calcium transport pump in the Ehrlich ascites tumor cell plasma membrane. Arch. of Biochem. and Biophys. 226(2): 618-628.

KOWARSKI, S. & SCHACHTER, D. (1973) Vitamin D and adenosine triphosphatase dependent on divalent cations in rat intestinal mucosa. J.Clin.Invest. 52: 2767-2773.

LANE, S.M. & LAWSON, D.E.M. (1978) Differantiation of the changes in alkaline phosphatase from calcium ion-activated adenosine triphosphatase activities associated with increased calcium absorption in chick intestine. Biochem.J. 174: 1067-1070.

LARSEN, F.L., HINDS, T.R. & VINCENZI, F.F. (1978) On the red blood cell Ca^{2+} pump: an estimate of stoichiometry. J.Membrane Biol. 41: 361-376.

LYNCH, T.J. & CHENNY, W.Y. (1979) Human erythrocyte Ca^{2+}-Mg^{2+}-ATPase: mechanism of stimulation by Ca^{2+}. Arch.Biochem.Biophys. 194: 165-170.

MA, S.W.Y., SHAMI, Y., MESSER, H.H. & COPP, D.H. (1974) Properties of Ca^{2+}-ATPase from the gill of rainbow trout *(Salmo gairdneri)*. Biochim.Biophys. Acta 345: 243-251.

MAETZ, J. & BORNANCIN, M. (1975) Biochemical and biophysical aspects of salt excretion by chloride cells in teleosts. Fortschr.Zool. 23: 322-362.

MARTIN, D.L., MELANCON, M.J.Jr. & DeLUCA, H.F. (1969) Vitamin D-stimulated, calcium-dependent adenosine triphosphatase from brush border of rat small intestine. Biochem.Biophys.Res.Commun. 35: 819-823.

MIRCHEFF, A.K. & WRIGHT, E.M. (1976) Analytical isolation of Na,K-ATPase rich membranes and the distribution of enzyme activities. J.Membrane Biol. 28: 309-333.

MIRCHEFF, A.K., WALLING, M.W., VAN OS, C.H. & WRIGHT, E.M. (1977) Distribution of alkaline phosphatase and Ca-ATPase in intestinal epithelial cell plasma membranes: differential response to $1,25(OH)_2D_3$. (Edited by Norman, A.W., Schaefer, K., Coburn, J.W., DeLuca, H.F., Fraser, D., Grigoleit, H.G., Herrath, D.V.). In: Vitamin D: biochemical, chemical and clinical aspects related to calcium metabolism. Walter de Gruyter, Berlin, 281-283.

MIRCHEFF, A.K., SACHS, G., HANNA, S.D., LABINER, C.S., RABON, E., DOUGLAS, A.P., WALLING, M.W. & WRIGHT, E.M. (1979) Highly purified baso-lateral plasma membranes from rat duodenum. Physical criteria for purity. J.Membrane Biol. 50: 343-363.

MOON, T.W. (1978) The characterization of ATPases from the gill of the osteoglossid *Osteoglossu bicirrhosum* (aruma). Can.J.Zool. 56: 795-800.

PERSHADSING, H.A., McDONALD, M.L., LANDT, M., BRY, C.G., LACY, P.E. & McDONALD, J.M. (1980) Ca^{2+}-activated ATPase and ATP-dependent calmodulin stimulated Ca^{2+}-transport in islet cell plasma membrane. Nature 288: 492-495.

REED, K.C. & BYGRAVE, F.L. (1975) Methodology for *in vitro* studies of Ca^{2+}-transport. Analyt.Biochem. 67: 44-54.

SCHARFF, O. (1979) Comparison between measured and calculated concentrations of calcium ions in buffers. Anal.Chim.Acta 109: 291-305.

SCHATZMANN, H.J. (1982) The plasma membrane calcium pump of erythrocytes and other animal cells. In: Carafoli, E. (ed) Membrane transport of calcium. Academic Press, London, 41-108.

SHEPARD, K.L. (1981) The activity and characteristics of the Ca^{2+}-ATPase of fish gills in relation to environmental calcium concentrations. J.exp.Biol. 90: 115-121.

SO, Y.P. & FENWICK, J.C. (1977) Relationship between net ^{45}Calcium influx across a perfused isolated eel gill and the development of post-stanniectomy hypercalcemia. J.exp.Zool. 200: 259-264.

VAN BELLE, H. (1981) R24571: a potent inhibitor of calmodulin-activated enzymes. Cell Calcium 2: 483-493.

VAN OS, C.H. & GHIJSEN, W.E.J.M. (1981) High-affinity Ca^{2+}-ATPase in basolateral plasma membranes of rat duodenum and kidney cortex. In: Bronner, F., Peterlik, M. (eds) Calcium and phosphate transport across membranes. Academic Press, New York, 159-162.

VAN OS, C.H. & GHIJSEN, W.E.J.M. (1982) Calcium transport mechanisms in rat duodenal basolateral plasma membranes: effects of $1,25(OH)_2D_3$. In: Norman, A.W., Schaefer, K., Herrath, D.V., Grigoleit, H.G. (eds) Vitamin D: chemical, bio-

chemical and clinical endocrinology of calcium metabolism. Walter de Gruyter, New York, 295-297.
VINCENZI, F.F. & LARSEN, F.L. (1980) The plasma membrane calcium pump: regulation by a soluble Ca^{2+}-binding protein. Am.J.Physiol. 233: 488-494.
WAISMAN, D.M., GIBLE, J.M., GOODMAN, D.B.P. & RASMUSSEN, H. (1981) Studies of the Ca^{2+}-transport mechanism of human erythrocyte inside-out plasma membrane vesicles. I. Regulation of the Ca^{2+}-pump by calmodulin. J.Biol.Chem. 256: 409-414.
WASSERMAN, R.H., BRINDAK, M.E., MEYER, S.A. & FULLMER, C.S. (1982) Calcium absorption and $1,25(OH)_2D_3$: studies with rachitic and partially vitamin-D-repleted chicks. In: Norman, A.W., Schaefer, K., Herrath, D.V., Grigoleit, H.G. (eds) Vitamin D: chemical, biochemical and clinical endocrinology of calcium metabolism. Walter de Gruyter, New York, 275-281.
WENDELAAR BONGA, S.E. & FLIK, G. (1982) Prolactin and calcium metabolism in a teleost fish, *Sarotherodon mossambicus*. In: Oguro, C. Pang, P.K.T. (eds) Comparative endocrinology of calcium regulation. Japan Scientific Societies Press, Tokyo, 21-26.

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Ca^{2+}-DEPENDENT PHOSPHATASE AND Ca^{2+}-DEPENDENT ATPase ACTIVITIES IN PLASMA MEMBRANES OF EEL GILL EPITHELIUM. III. STIMULATION OF BRANCHIAL HIGH-AFFINITY Ca^{2+}-ATPase ACTIVITY DURING PROLACTIN INDUCED HYPERCALCEMIA IN AMERICAN EELS.

ABSTRACT

1. Infusions of ovine prolactin for 10 days induced hypercalcemia in unfed American eels, *Anguilla rostrata* LeSueur, that tentatively was related to stimulation of branchial Ca^{2+}-uptake mechanisms.
2. Analysis of ATPase activities in the plasma membranes of the branchial epithelium in prolactin treated eels showed a specific stimulation of high-affinity Ca^{2+}-ATPase.
3. The results of this study form further evidence that the high-affinity Ca^{2+}-ATPase activity represents the Ca^{2+}-pump of the branchial epithelium.

INTRODUCTION

Fish can regulate their serum calcium levels with great precision (Bailey & Fenwick, 1975; Copp & Ma, 1978) and this ability must be attributed, at least in part, to the capacity of their gills to absorb calcium directly from their aquatic environment (Berg, 1968; Simmons, 1971; Simkiss, 1974; So & Fenwick, 1977; Payan *et al.*, 1981). Unfortunately, there is a paucity of information concerning the mechanisms of this branchial calcium uptake.

Recently we reported the simultaneous occurrence of non-specific phosphatase activity and high-affinity Ca^{2+}-ATPase activity in American eel gill plasma membranes (Flik *et al.*, 1983; Flik *et al.*, 1984). Further, we equated the - heterogeneous - non-specific phosphatase activity with Ca^{2+}-activated ATPase activity which has been reported to be present in the gills of many teleostean species and which has been described as related to calcium transport (Ma *et al.*, 1974; Fenwick, 1976; Moon, 1978; Fenwick, 1979; Ho & Chan, 1980; Doneen, 1981). However, the characteristics of this activity more closely resemble those of an alkaline phosphatase rather than a transport Ca^{2+}-ATPase (Ghijsen *et al.*, 1980). On the other hand, we identified the branchial high-affinity Ca^{2+}-ATPase as a

calmodulin-sensitive ATPase that was stimulated by intracellular Ca^{2+}-concentrations in the presence of excess Mg^{2+} (Flik *et al.*, 1984). These characteristics suggested that it was this latter activity which is associated with the branchial Ca^{2+}-pump in fish.

The present study was directed towards testing the effect of ovine prolactin on both the Ca^{2+}-dependent phosphatase and the high-affinity Ca^{2+}-ATPase in American eel gill plasma membranes to determine which, if either, of the activities respond to prolactin treatment.

Prolactin is known to induce hypercalcemia in several species of fish (Pang *et al.*, 1978; Wendelaar Bonga & Flik, 1982), including American eel (Ma & Copp, 1978). It was further reported that ovine prolactin enhanced calcium influx in perfused American eel gills (Ma & Copp, 1981). The rationale behind our present study was that if prolactin stimulates gill calcium absorption it could stimulate enzymic activities associated with active calcium transport.

MATERIALS AND METHODS

Adult female yellow eels, *Anguilla rostrata* LeSueur, with an average body weight of 1.7 kg were obtained from a commercial dealer in Québec City, Québec, Canada. The eels were held in running dechlorinated Ottawa tapwater (0.45 mM Ca^{2+}, 12°C) with 16 h of light alternating with 8 h of darkness. The animals were not fed.

Hormone treatment

Ovine prolactin was kindly supplied by the Hormone Distribution Agency of the National Institutes of Health (Bethesda, Md.) and was administered continuously for ten days by means of Alzet osmotic minipumps implanted intraperitoneally. The dosage was 0.1 U.g $fish^{-1}.day^{-1}$, dissolved in 0.05 N HCl. Controls received equivalent amounts of solvent. Ovine prolactin, at the doses used in this study, generally mimics homologous prolactin as far as its effects on osmoregulation and Ca^{2+}-metabolism of fish (Wendelaar Bonga & Van der Meij, 1980). At the end of the experiments the infusion rates of the pumps were checked by measuring the pump contents (maximum infusion duration of the pumps approximated 19 days at 12°C) and a maximum deviation of 10% was accepted. The stability of the hormone preparation was checked electrophoretically by comparison of freshly dissolved prolactin with prolactin recovered from the pumps at the end of the experiments. Silver stained electrophoretographs did not reveal

any differences. Control and prolactin treated animals were individually marked by fin clipping and were kept in the same tank during the experiment.

At the end of the experimental period the eels were anesthetized in a Tris-buffered (pH 7.4) MS222 solution and the blood was collected into heparinized tuberculin syringes by direct cardiac puncture. After centrifugation of the blood, total plasma Ca was estimated with a commercial Ca kit (Sigma), Mg analyzed by atomic absorption spectrophotometry, and Na and K by flame emission spectrophotometry; inorganic phosphate was analyzed by a modification of the method of Fiske and Subbarow (Fenwick, 1976).

Isolation of branchial plasma membranes and enzyme assays

Plasma membranes were isolated and assayed for protein, succinic dehydrogenase, Na^+/K^+-ATPase, Ca∿ATP-phosphatase and high-affinity Ca^{2+}-ATPase activities as described previously (Flik *et al.*, 1983; Flik *et al.*, 1984). Statistical analysis of the results was carried out applying Student's t-test (two-sided, α=5%).

RESULTS

Blood plasma analysis

Prolactin treatment of eels increased plasma calcium levels significantly from 2.84 ± 0.17 mM in controls to 3.40 ± 0.20 mM ($P < 0.01$). No significant differences in plasma Na, K or P_i levels were observed (Table I).

Table I. Effects of ovine prolactin on blood plasma mineral composition. Plasma mineral content is expressed in mM. Mean values ± s.d. are given, with the number of animals in parentheses.

	Controls (5)	Experimentals (4)
Na	141.5 ± 14.1	135.8 ± 6.9
K	1.89 ± 0.06	1.84 ± 0.19
Mg	0.91 ± 0.05	0.50 ± 0.12*
Ca	2.84 ± 0.17	3.40 ± 0.20*
P_i	1.35 ± 0.13	1.12 ± 0.20

* : $P < 0.01$

Enzyme analysis

Prolactin treated eels showed a statistically significant increase in high-affinity Ca^{2+}-ATPase specific activity (measured as the 1 µM Ca^{2+}-induced Mg∿ATP hydrolysis). Neither Na^+/K^+-ATPase nor Ca∿ATP-phosphatase activities were affected by this treatment. Succinic dehydrogenase specific activities in whole tissue homogenates and in the enriched plasma membrane fractions of control and prolactin treated fish did not differ significantly and were similar to values reported for untreated eels (Flik *et al.*, 1983). The amounts of total protein extracted from the gills and recovered in the plasma membrane fraction were not significantly different for control and experimental fish.

Calculated total branchial high-affinity Ca^{2+}-ATPase activities were 13.4 µmol P_i per hour for controls and 21.0 µmol P_i per hour for prolactin treated eels (57% stimulation). Total Na^+/K^+-ATPase activities were 157.2 and 170.8 µmol P_i per hour for controls and experimentals, respectively. Ratios for total Na^+/K^+-ATPase to total high-affinity Ca^{2+}-ATPase were 11.7 for controls and 8.1 for prolactin treated eels, indicating a 44% stimulation of high-affinity Ca^{2+}-ATPase activity relative to Na^+/K^+-ATPase activity in branchial plasma membranes after prolactin treatment (Table II).

Table II. Effects of ten days infusion of ovine prolactin on plasma membrane ATPase and phosphatase activities in the gills. Specific activities are expressed as µmol $P_i.h^{-1}.mg\ protein^{-1}$. Mean values ± s.d. are given with the number of animals in parentheses.

	Controls (4)	Experimentals (4)	Statistical Significance
High-affinity Ca^{2+}-ATPase	2.56 ± 0.50	4.94 ± 0.58	$P < 0.01$
Na^+/K^+-ATPase	75.7 ± 20.0	74.5 ± 15.1	N.S.
Ca∿ATP-phosphatase	81.2 ± 10.0	80.3 ± 7.4	N.S.

DISCUSSION

Prolactin and blood plasma mineral composition

Ovine prolactin induced hypercalcemia in fresh water yellow eels and this result agrees with earlier findings from killifish (Pang *et al.*, 1978), sticklebacks (Wendelaar Bonga *et al.*, 1978), the tilapia *Sarotherodon mossambicus* (Wendelaar Bonga & Flik, 1982; Wendelaar Bonga *et al.*, 1983) and American eels

(Ma & Copp, 1981). In addition, prolactin induced hypomagnesemia, a phenomenon also reported earlier for tilapia (Wendelaar Bonga *et al.*, 1983). In mammals such a hypomagnesemia is considered to be a direct effect of the concomittant hypercalcemia (Ebel & Guenther, 1980). Prolactin did not affect plasma Na, K or P_i levels, which indicates the specific nature of the hypercalcemic action of prolactin under these conditions. The absence of a concommittant hyperphosphatemia further suggested that the prolactin induced hypercalcemia did not result from mobilization of Ca^{2+} from bone minerals (apatites). Ma & Copp (1981) have shown for American eels, the species used in this study, that this effect can be ascribed to stimulation by prolactin of Ca^{2+}-uptake in the gills.

Prolactin and branchial ATPase activities

Prolactin enhanced high-affinity Ca^{2+}-ATPase activities in the plasma membranes of eel branchial epithelium, which suggests that prolactin may influence Ca-metabolism by activating a Ca^{2+}-pump in the gills. Prolactin did not stimulate the non-specific Ca∿ATP-phosphatase activity. We take these observations as further evidence that it is the high-affinity Ca^{2+}-ATPase and not the non-specific phosphatase which functions as a Ca^{2+}-transporting enzyme. The recovery of branchial total Na^+/K^+-ATPase, total Ca∿ATP-phosphatase and total protein was similar in prolactin treated and control eels. The absence of an effect on either the Ca∿ATP-phosphatase or the Na^+/K^+-ATPase activities indicates that the action of prolactin on the gills was not of a general trophic character. This is also supported by the fact that the ratios of total Na^+/K^+-ATPase to total high-affinity Ca^{2+}-ATPase differed between prolactin treated and control eels. The decrease of the ratio from 11.7 in the controls to 8.1 in prolactin treated eels suggested a specific induction of high-affinity Ca^{2+}-ATPase in branchial plasma membranes.

Interestingly, our results show some close similarities with data published by Ghijsen & Van Os (1982) on rat intestine. These authors reported that $1\alpha,25(OH)_2$-vitamin D_3 specifically stimulated a transport Ca^{2+}-ATPase, that resembles the eel branchial Ca^{2+}-ATPase closely, but not Na^+/K^+-ATPase to transport Ca^{2+}-ATPase activities amounted to 11.6 in the controls and 7.1 in $1\alpha,25(OH)_2$ vitamin D_3-repleted animals. Using rachitic rats, Pahuja & DeLuca (1981) showed that also prolactin directly influences intestinal Ca absorption in a way comparable to $1\alpha,25(OH)_2$ vitamin D_3; these authors concluded that the action of prolactin was independent of the vitamin D endocrine system.
In conclusion, it is our opinion that prolactin is involved not only in the con-

trol of branchial permeability for water and monovalent ions (Wendelaar Bonga & Van der Meij, 1981; Wendelaar Bonga *et al.*, 1983), but also in the regulation of the branchial Ca^{2+}-pump. This contention contributes new to the discussion on the role of prolactin in hydromineral balance of freshwater teleosts.

REFERENCES

BAILEY, J.R. & FENWICK, J.C. (1975) Effect of angiotensin II and the corpuscles of Stannius extract on total and ionic plasma calcium levels and blood pressure in intact eels *(Anguilla rostrata* LeSueur). Gen.J.Zool. 53: 630-633.

BERG, A. (1968) Studies on the metabolism of calcium and strontium in fresh water fish. I. Relative contribution of direct and intestinal absorption. Me.Inst.Ital.Idrobiol. 23: 161-196.

COPP, D.H. & MA, S.W.Y. (1978) Endocrine control of calcium matabolism in vertebrates. In: Gaillard, P.J. & Boer, H.H. (eds) Comparative Endocrinology, Elsevier/North Holland Biomedical Press, Amsterdam, 243-253.

DONEEN, B.A. (1981) Effects of adaptation to sea water, 170% sea water and to fresh water on activities and subcellular distribution of branchial Na^+/K^+-ATPase, low- and high-affinity Ca^{2+}-ATPase, and ouabain-insensitive ATPase in *Gillichthys mirabilis*. J.Comp.Physiol. 145: 51-61.

EBEL, H. & GUENTHER, T. (1980) Magnesium metabolism: a review. J.Clin.Chem.Clin. Biochem. 18: 257-270.

FENWICK, J.C. (1976) Effects of stanniectomy on calcium activated adenosinetriphosphatase activity in the gills of fresh water adapted North American eels, *Anguilla rostrata* LeSueur. Gen.Comp.Endocrinol. 29: 383-387.

FENWICK, J.C. (1979) Ca^{2+}-activated adenosinetriphosphatase activity in the gills of fresh water and sea water adapted eels *(Anguilla rostrata)*. Comp. Biochem.Physiol. 62B: 67-70.

FLIK, G., REINTJENS, F.M.J., STIKKELBROECK, J. & FENWICK, J.C. (1982) 1,25-Vitamin D_3 and calcium transport in the gut of tilapia *(Sarotherodon mossambicus)*. J.Endocrinol. 94: 40P.

FLIK, G., WENDELAAR BONGA, S.E. & FENWICK, J.C. (1983) Ca^{2+}-dependent phosphatase and ATPase activities in eel gill plasma membranes- I. Identification of Ca^{2+}-activated ATPase activities with non-specific phosphatase activities. Comp.Biochem.Physiol. 76B: 745-754.

FLIK, G., WENDELAAR BONGA, S.E. & FENWICK, J.C. (1984) Ca^{2+}-dependent phosphatase and Ca^{2+}-dependent ATPase activities in eel gill plasma membranes- II. Evidence for a high-affinity Ca^{2+}-ATPase. Comp.Biochem.Physiol. In press.

GHIJSEN, W.E.J.M., DE JONG, M.D. & VAN OS, C.H. (1980) Dissociation between Ca^{2+}-ATPase and alkaline phosphatase activities in plasma membranes of rat duodenum. Biochim.Biophys.Acta 559: 538-551.

GHIJSEN, W.E.J.M. & VAN OS, C.H. (1982) $1\alpha,25(OH)_2$vitamin D_3 regulates ATP-dependent calcium transport in basolateral plasma membranes of rat enterocytes. Biochim.Biophys.Acta 689: 170-172.

HO, S.M. & CHAN, D.K.O. (1980) Branchial ATPases and ionic transport in the eel *Anguilla japonica* - II. Ca^{2+}-ATPase. Comp.Biochem.Physiol. 67B: 639-645.

MA, S.W.Y., SHAMI, Y., MESSER, H.H. & COPP, D.H. (1974) Properties of Ca^{2+}-ATPase from the gill of rainbow trout *(Salmo gairdneri)*. Biochim.Biophys.Acta 345: 243-251.

MA, S.W.Y. & COPP, D.H. (1981) Prolactin and calcium metabolism in teleosts. In: Cohn, D.V., Talmage, R.V. & Matthews, J.L. (eds) Hormonal control of Calcium Metabolism. Excerpta Medica, Amsterdam, 423.

MOON, T.W. (1978) The characterization of ATPases from the gills of the osteoglossid *Osteoglossu bicirrhosum* (aruana). Can.J.Zool. 56: 795-800.
PAHUJA, D.N. & DeLUCA, H.F. (1981) Stimulation of intestinal calcium transport and bone calcium mobilization by prolactin in vitamin D-deficient rats. Science 214(27): 1038-1039.
PANG, P.K.T., SCHREIBMAN, M.P., BALBONTIN, F. & PANG, R.K. (1978) Prolactin and pituitary control of calcium regulation in the killifish, *Fundulus heteroclitus*. Gen.Comp.Endocrinol. 36: 306-316.
PAYAN, P., MAYER-GOSTAN, N. & PANG, P.K.T. (1981) Site of calcium uptake in the fresh water trout gill. J.Exp.Zool. 216: 345-347.
SIMKISS, K. (1974) Calcium metabolism of fish in relation to ageing. In: Begenal, T.B. (ed) Ageing of Fish. Unwin, Old Woking, England, 1-12.
SIMMONS, D.J. (1971) Calcium and skeletal tissue physiology in teleosts fishes. Clin.Orthop.Related.Res. 76: 244-280.
SO, Y.P. & FENWICK, J.C. (1977) Relationship between net ^{45}Ca influx across a perfused isolated eel gill and the development of poststanniectomy hypercalcemia. J.Exp.Zool. 200: 259-264.
WENDELAAR BONGA, S.E. & GREVEN, J.A.A. (1978) The relationship between prolactin cell activity, environmental calcium and plasma calcium in the teleost *Gasterosteus aculeatus*. Observations on Stanniectomized fish. Gen.Comp.Endocrinol. 36: 90-101.
WENDELAAR BONGA, S.E. & VAN DER MEIJ, J.C.A. (1981) The effect of ambient calcium on prolactin cell activity and plasma electrolytes in *Sarotheodon mossambicus* (tilapia mossambica). Gen.Comp.Endocrinol. 40: 391-401.
WENDELAAR BONGA, S.E. & FLIK, G. (1982) Prolactin and calcium metabolism in a teleost fish. In: Oguro, C. & Pang, P.K.T. (eds) Comparative Endocrinology of Calcium Regulation. Tokyo, Japan Scientific Societies Press, 21-26.
WENDELAAR BONGA, S.E., LOEWIK, C.J.M. & VAN DER MEIJ, J.C.A. (1983) Effects of external Mg^{2+} and Ca^{2+} on branchial osmotic water permeability and prolactin secretion in the teleost fish *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. 52: 222-231.

STUDIES ON THE BRANCHIAL EPITHELIUM OF THE TILAPIA *OREOCHROMIS MOSSAMBICUS*, ADAPTED TO FRESH WATER: EVIDENCE FOR ACTIVE Ca^{2+}-TRANSPORT MECHANISMS IN PLASMA MEMBRANES

ABSTRACT

1. A high-affinity Ca^{2+}-ATPase activity was demonstrated among the phosphatase activities in plasma membranes of tilapia branchial epithelium; its characteristics ($K_{0.5}$ = 0.063 µM Ca^{2+}, V_{max} = 6.02 µmolP_i/h.mg protein at 37°C) resemble those of Ca^{2+}-translocating enzymes present in mammalian erythrocytes or enterocytes.
2. The ratio of this Ca^{2+}-ATPase activity to Na^+/K^+-ATPase activity was 1:20.4, a proportion identical to the one reported for these enzyme activities in eel gill plasma membranes.
3. Radioimmunoassayable calmodulin was demonstrated in the Ca^{2+}-ATPase containing membrane fraction.
4. ATP-dependent Ca^{2+}-transport was demonstrated in tight-vesicle preparations of the branchial cell membranes; the preparation consisted of 30% inside-out and 44% right-side-out vesicles, and included 26% leaky vesicles; the characteristics of the active Ca^{2+}-transport activity are consistent with a Ca^{2+}-extrusion mechanism involving high-affinity Ca^{2+}-ATPase activity.
5. The branchial Ca^{2+}-transport activity per fish, as calculated on the basis of the transport activity determined for the vesicle preparation, is in the order of the branchial Ca^{2+}-influx rates observed *in vivo*.
6. The data provide the first biochemical evidence for active Ca^{2+}-transport in plasma membranes of branchial epithelium.
7. A model is presented for the mechanism of active transepithelial Ca^{2+}-transport in fish gills.

INTRODUCTION

In previous reports (Flik *et al.*, 1983, 1984a and 1984b) evidence was given for the presence of high-affinity Ca^{2+}-ATPase activity in plasma membranes of

branchial epithelium of fresh water American eels. Characteristics of the enzyme activity are: an affinity for Ca^{2+} (0.22 μM Ca^{2+}) in the intracellular Ca^{2+}-concentration range, ATP-preference, and calmodulin-dependency. These characteristics are shared with established mammalian Ca^{2+}-ATPases involved in Ca^{2+}-transport. Induction of high-affinity Ca^{2+}-ATPase in eel gill plasma membranes was observed after treatment of eels with the hypercalcemic hormone prolactin, which further indicated an involvement of this enzyme in active uptake of Ca^{2+} from the water. To our knowledge, these are the only reports on a Ca^{2+}-ATPase activity in fish that fulfils criteria for an enzyme to be directly involved in trans-cellular Ca^{2+}-transport.

The present studies were undertaken to analyze plasma membranes of the gills of the teleost fish tilapia *(Oreochromis mossambicus)* for high-affinity Ca^{2+}-ATPase activity and to assess whether Ca^{2+}-transporting activity is present in plasma membranes, that may be connected with this enzyme activity. Ca^{2+}-induced Mg∿ATP-hydrolysis was studied in Na^{+}/K^{+}-ATPase-enriched membrane fractions isolated from branchial epithelium. ATP-dependent Ca^{2+}-transport activity was determined in tight-vesicle preparations of these membranes. As a first step to verify whether these Ca^{2+}-dependent processes are calmodulin-dependent, as we have shown before for the high-affinity Ca^{2+}-ATPase activity in eel gills (Flik *et al.*, 1984a), the presence and concentration of calmodulin in the gill membrane fraction were investigated. Membrane orientation in resealed vesicles was determined on the basis of the sidedness of acetylcholine esterase and glyceraldehyde-3-phosphate dehydrogenase. The total branchial Ca^{2+}-transport capacity *in vitro* was calculated and compared to branchial Ca^{2+}-influx rates as determined *in vivo*.

MATERIALS AND METHODS

Male tilapia, *Oreochromis mossambicus*, were used in all experiments. Body weights ranged from 10-30 g. Fresh water specimens were obtained from laboratory stock, kept at 28°C under conditions as described previously (Wendelaar Bonga & Van der Meij, 1980). The Ca^{2+}-concentration of the water was 0.8 mM.

Reagent grade chemicals were purchased from Sigma. Ultrapure water was used in enzyme assays and Ca^{2+}-transport studies that involved the use of Ca^{2+}-buffers.

Isolation of plasma membranes

To collect branchial epithelium, animals were killed by transection of the spinal cord, the branchial apparatus was removed and the epithelium scraped off onto an ice-cold glass plate. Scrapings were collected in an isotonic buffer containing (mM): sorbitol (250), NaCl (12.5), imidazol/histidine (5.0,pH 7.5), PMSF (0.2), dithiothreitol (0.1), aprotinin (100 U/ml) and EDTA (0.1). Using this buffer and a loosely-fitting dounce homogenizer allows disruption of the branchial epithelium but leaves erythrocytes intact. The latter were separated from the branchial epithelial membrane fragments by a 550 *g*. 10 min centrifuge run. Light-microscopic examination of the pellet obtained after this centrifugation step revealed a homogeneous population of intact red blood cells and nuclei. Membranes of the disrupted epithelium were collected by centrifugation of the supernatant (designated H_0) remaining after separation of the erythrocytes, yielding a pellet (P_0) and a supernatant which was designated as the cytosolic fraction (S_0). P_0 was further purified by differential centrifugation as described for eel gill epithelial membranes (Flik *et al.*, 1983), yielding a plasma membrane fraction called P_3 (Method I), or by isopycnic centrifugation on a discontinuous sorbitol gradient, yielding a plasma membrane fraction called P_3' (Method II). For gradient centrifugation P_0 was resuspended (100 strokes) with a douncer in 21% sorbitol in homogenization buffer; a 3-ml aliquot of this suspension was layered on two blocks of 4.5 ml of 43% and 36% sorbitol and run for 200 K*g*. 180 min at 4°C (Beckman L8-80, SW40 ti). The band appearing at the 43%/36% sorbitol interface was collected, pelleted after dilution with sorbitol free buffer by ultra-centrifugation (300 K*g*. 30 min; SW50 ti) and appeared to consist of membranes highly enriched in Na^+/K^+-ATPase. Plasma membranes were resuspended in the basic assay buffer, divided in portions as required for the assays, frozen in liquid nitrogen and stored at -80°C for a maximum of 1 week. For the preparation of sealed plasma membrane vesicles, EDTA was omitted after the initial homogenization step. Membranes to be used for Ca^{2+}-transport studies were resealed in a buffer containing (mM): KCl (150), $MgCl_2$ (5) and Hepes/Tris (30, pH 7.4), according to the method of Ghijsen *et al.* (1982). Membranes for orientation and Ca^{2+}-transport studies were used immediately after isolation without being frozen.

Assays and assay media

Protein, Na^+/K^+-ATPase, succinic acid dehydrogenase (SDH) and Ca^{2+}-stimulated ATPase activities were determined as described in detail elsewhere (Flik *et al.*,

1983, 1984a). Calmodulin was determined using a commercial ^{125}I-radioimmunoassay kit (New England Nuclear, NEK-18) with a highly specific antibody raised in sheep against non-derivatized rat testis calmodulin. Membrane fractions (H_0, P_3') were assayed without further purification. The cytosolic fraction (S_0, the supernatant obtained by ultracentrifugation of disrupted branchial epithelium) was partly purified as suggested by Teo *et al.* (1973) and as described in detail for the purification of calmodulin from fish mucus (Flik *et al.*, 1984c).

Membrane impermeability of resealed plasma membrane vesicles was tested by the exclusion of substrate to membrane-bound enzymes with established sidedness. Acetylcholine esterase (exoenzyme) and glyceraldehyde-3-phosphate dehydrogenase (GPDH, endoenzyme) activities were determined in resealed vesicle preparations treated with varying concentrations of detergent (0-0.2% v/v Triton X-100), as suggested for erythrocyte membranes by Steck & Kant (1974). No modifications were introduced in these assays. Initial enzyme velocities (upon addition of substrate) in the presence of optimal detergent concentrations were equated with 100% accessibility. Enzyme activities in the absence of detergent were expressed as percentages of the maximum activities observed. From the accessibility percentages for the two enzymes we calculated the percentages inside-out (IOV), right-side-out (ROV) and leaky vesicles of a membrane preparation.

Ca^{2+}-transport activity in gill plasma membranes was determined as ATP-dependent ^{45}Ca-accumulation over a 2-5 min period at 37°C in resealed vesicles, according to the method of Ghijsen *et al.* (1982). The amount of protein per filter was 7.4 µg BSA equivalents.

Statistics and calculations

Values are expressed as means ± standard deviations. Statistical analysis of differences between mean values was carried out applying Students *t*-test for unpaired observations (α=5%). Significance was accepted at the 2% level. Determination of $K_{0.5}$ for Ca^{2+} and V_{max}-values from Eadie-Hofstee plots of Ca^{2+}-stimulated ATPase and transport activities in plasma membranes was performed according to Borst Pauwels (1973). Linear regression analysis was based on the least-squares method.

Plasma membrane isolation

In Table I the percentages recovery of protein, Na^+/K^+-ATPase and SDH activities are presented for the isolation of plasma membrane enriched fractions (P_3 and P_3'). The P_3-fraction contained 2.82% of the initial protein, 4.52% of the initial Na^+/K^+-ATPase (used as plasma membrane marker). In those cases where P_3'-fractions were prepared, 0.36% of the initial membrane protein, 4.38% of the initial Na^+/K^+-ATPase and less than 1% of the initial SDH activities were recovered. During these procedures the Na^+/K^+-ATPase specific activities for the P_3- and the P_3'-fractions increased approximately 3.5 and 12 times, respectively.

Table I. Percentage recoveries and specific activities of marker enzymes in tilapia gill plasma membranes.

Recoveries were expressed as percentages of the total enzyme activities in the initial homogenate (H_0). Na^+/K^+-ATPase specific activities were expressed in µmol P_i/h.mg protein at 37°C. Mean values ± S.E. are given; n=5.

	H_0		P_3		P_3'	
	% Recovery	V_{spec}	% Recovery	V_{spec}	% Recovery	V_{spec}
Protein	100	-	2.82 ± 0.34	-	0.36 ± 0.14	-
Na^+/K^+-ATPase	100	8.1 ± 2.9	4.52 ± 0.91	30.9 ± 1.9	4.38 ± 1.77	123.3 ± 33.0
SDH	100	-	1.21 ± 0.23	-	<1	-

To exclude mitochondrial ATPase activity in assays for plasma membrane Ca^{2+}-stimulated ATPase activities we routinely added oligomycin B (5 µg/ml) and sodium azide (5 mM) to the pertinent assay media.

In Table II Na^+/K^+-ATPase specific activities are given for P_3-fractions isolated in the presence or absence of EDTA. Na^+/K^+-ATPase specific activities for P_3-fractions isolated in the presence of EDTA amounted to 30.9 ± 1.9 µmol P_i/h.mg protein at 37°C and were not affected by detergent treatment. Na^+/K^+-ATPase activities in membranes isolated in the absence of EDTA increased by a factor of 2.2 when pretreated with 0.1% v/v Triton X-100 for 1 min at 37°C, and reached levels similar to those observed in membranes isolated in the presence of EDTA. The activation of the extrinsic Na^+/K^+-ATPase indicates that

isolating membranes in the absence of EDTA yields a preparation that at least partially consists of resealed vesicles. Such resealed plasma membrane vesicle preparations showed 56% acetylcholine esterase accessibility and 70% GPDH accessibility, which indicates that the preparation consists of 26% leaky, 30% inside-out, and 44% right-side-out vesicles. Consistent with these results was the observation by transmission electron microscopy of a homogeneous population of circular bilayer membranes with only a low incidence of membrane fragments (results not shown).

Table II. Vesicle tightness and membrane orientation.

P_3-fractions isolated in the presence or absence of EDTA were analyzed for Na^+/K^+-ATPase, acetylcholine esterase and GPDH activity after treatment with buffered Triton X-100 (0.1 - 0.2% v/v) or buffer only (controls). Enzyme activities determined in the presence of optimal concentrations of the detergent were designated 100%. Values for Na^+/K^+-ATPase specific activities (expressed in µmol P_i/h.mg protein, determined at 37°C) are given in parentheses. Mean values ± S.E. are given; n = number of determinations.

	Isolated + EDTA			Isolated - EDTA	
	n	Triton X-100	Controls	Triton X-100	Controls
Na^+/K^+-ATPase	5	100 (31.2±2.4)	99 (30.9±1.9)	100 (27.9±5.7)	45 (12.5±3.6)
Acetylcholine esterase	8	-	-	100	70±14
GPDH	8	-	-	100	56±11

Ca^{2+}-stimulated ATPase activity

In Fig. 1, Ca^{2+}-stimulated ATPase activities determined in P_3'-fractions are shown. Ca^{2+}-induced Mg∿ATP-hydrolysis represented by an Eadie-Hofstee plot revealed a high-affinity and a low-affinity ATPase activity. $K_{0.5}$- and V_{max}-values for the high-affinity component were calculated to 0.06 µM Ca^{2+} and 4.9% stimulation of the Mg∿ATP-hydrolysis observed in the absence of Ca^{2+}; for the low-affinity component these values were 14 µM Ca^{2+} and 10.7% stimulation, respectively. The kinetic parameters for the high-affinity component resemble closely those reported earlier for the high-affinity Ca^{2+}-ATPase of eel gill epithelium (Flik *et al.*, 1984a). The V_{max} for the tilapia high-affinity Ca^{2+}-ATPase in

P_3'-fractions amounted to 6.02 µmol P_i/h.mg protein at 37°C. The ratio of the Na^+/K^+-ATPase and the high-affinity Ca^{2+}-ATPase in these membranes was 20.4 to 1, a value identical to the one reported for eel gill plasma membranes.

Figure 1. Eadie-Hofstee plot of Ca^{2+} stimulated Mg∿ATP hydrolysis by plasma membranes of tilapia branchial epithelium. Enzymic activities (V) were expressed as percentage stimulation above background hydrolytic activity. Ca^{2+}-concentrations (S) are expressed in µM.

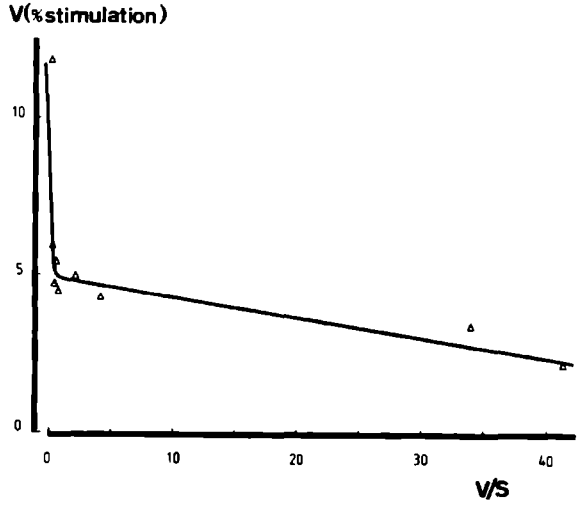


Table III. Calmodulin in tilapia branchial epithelium.

Calmodulin concentrations were determined by radioimmunoassay. H_0 refers to homogenate of branchial epithelium; S_0 refers to the supernatant obtained after ultracentrifugation of H_0, and represents the cytosol of the branchial epithelium. S_0 was partly purified, as described in "Materials and Methods". P_3' is the membrane fraction obtained after gradient-centrifugation; n=2.

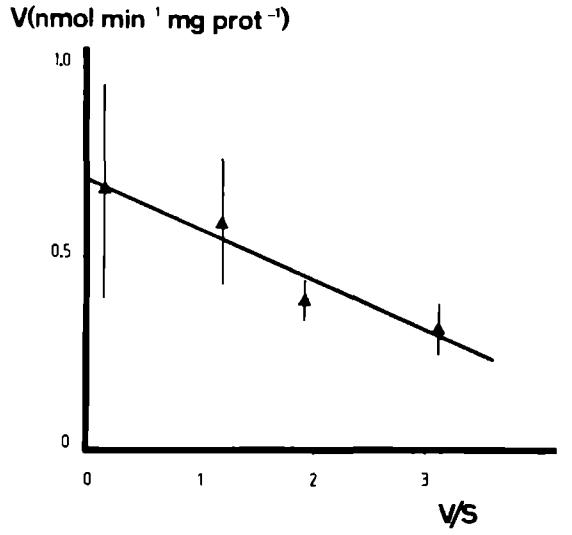
Fractions	Calmodulin (mg/g protein)
H_0	1.65 ± 0.05
S_0	1.98 ± 0.12*
P_3'	0.29 ± 0.03

* partly purified

Presence of calmodulin

In our reports on eel gill plasma membranes we concluded that the high-affinity Ca^{2+}-ATPase in these membranes is calmodulin-dependent and that the membrane fractions used contained significant amounts of calmodulin, even when low concentrations of EDTA (0.1 mM) were used throughout the isolation procedure (Flik *et al.*, 1984a). In the present study radioimmunoassayable calmodulin was found to be present not only in the cytosol fraction but also in the Ca^{2+}-ATPase containing plasma membranes, which were isolated in the presence of EDTA (Table III).

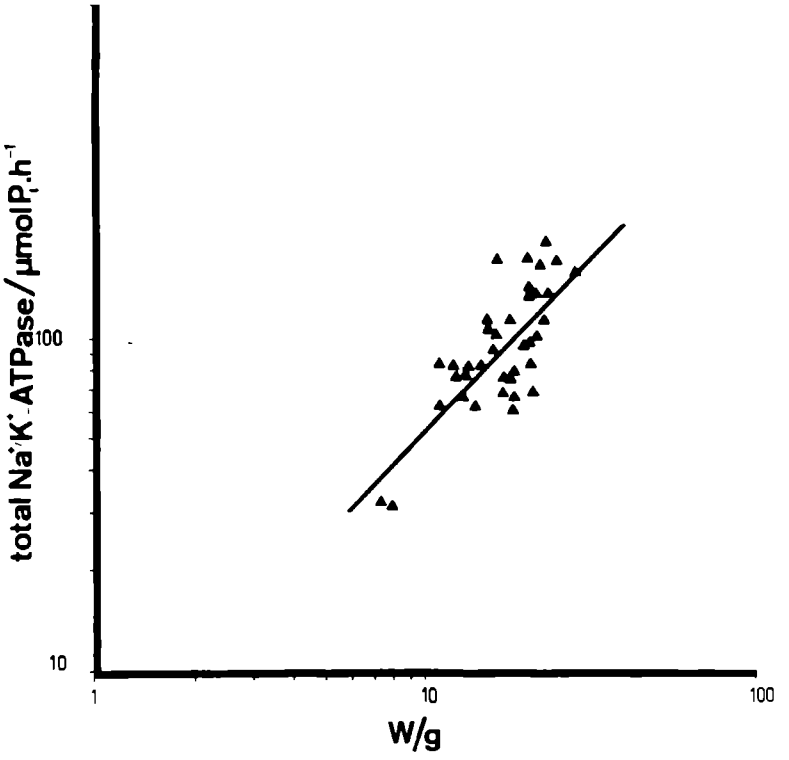
Figure 2. Eadie-Hofstee plot of Ca^{2+}-stimulated, ATP-dependent Ca^{2+}-transport in tight plasma membrane vesicles. Ca^{2+}-transport activity (V) was determined in 2-5 min incubations at 37°C. Ca^{2+}-concentrations were buffered and ranged from 0-5 µM (S). Ca^{2+}-transport was expressed in nmol/min.mg protein; results were not corrected for % IOV. $K_{0.5}$- and V_{max}-values were 0.13 µM Ca^{2+} and 0.7 nmol/min.mg protein.



Ca^{2+}-transport

ATP-dependent Ca^{2+}-transport in resealed plasma membrane vesicles of tilapia gill epithelium was assayed in the presence of up to 5 µM Ca^{2+}. An Eadie-Hofstee plot of the results revealed the existence of one affinity site for Ca^{2+} (Fig. 2). The calculated $K_{0.5}$ for Ca^{2+} was 0.13 µM and a V_{max} of 0.7 nmol Ca^{2+}/min.mg protein was found. This V_{max} is not corrected for % IOV.

Figure 3. Full-logarithmic plot of the relationship between total gill Na^+/K^+-ATPase and body weight (W) of freshwater tilapia. Total Na^+/K^+-ATPase was calculated as the product of Na^+/K^+-ATPase specific activity and total protein in H_0. Results were satisfactorily fitted by linear regression analysis yielding the relationship: total gill Na^+/K^+-ATPase = $4.74W^{1.035}$ µmol P_i/h at 37°C.



In fresh water tilapia, total gill Na^+/K^+-ATPase activity, calculated as the product of Na^+/K^+-ATPase specific activity and total protein, is directly related

to body weight (W) according to: total Na^+/K^+-ATPase = $4.74W^{1.035}$ µmol P_i/h (n=41; W: 7-30 g, r_o = 0.792, P < 0.001; Fig. 3). This relationship indicates that the amount of plasma membranes as well as the Na^+/K^+-ATPase-dependent Na^+-transport capacity of the gills is directly related to body weight. For a 20-g tilapia, total gill Na^+/K^+-ATPase is 105.3 µmol P_i/h. From an average specific activity of 8.1 µmol P_i/h.mg protein in H_0 and 2.82% recovery for protein in P_3 it follows that P_3 contains 0.367 mg protein. At a maximum Ca^{2+}-transport rate of 60 x 0.7 = 42 nmol Ca^{2+}/h.mg protein, 30% IOV and 0.367 mg protein in P_3, the Ca^{2+}-transporting capacity of this P_3 fraction is 51.4 nmol/h at 37°C. Assuming an activation energy of 57.9 kJ/mol as reported for the eel gill Ca^{2+}-ATPase (Flik *et al.*, 1984a) the Ca^{2+}-transporting capacity of the P_3-fraction comes to 26.2 nmol/h at 28°C, the temperature at which the tilapia were kept. Further, considering a 4.5% recovery of Na^+/K^+-ATPase in P_3, the total branchial Ca^{2+}-transporting capacity comes to 580 nmol/h at 28°C. Proceeding from a 20.4:1 ratio for Na^+/K^+-ATPase (Sarkadi, 1980), a total Na^+/K^+-ATPase activity of 105.3 µmol P_i/h for a 20-g fish and applying correction for temperature, a total Ca^{2+}-transporting capacity of 2633 nmol/h at 28°C is calculated.

DISCUSSION

We previously reported the existence of a high-affinity, calmodulin-dependent Ca^{2+}-ATPase activity in plasma membranes of eel gill epithelium (Flik *et al.*, 1984a). The present results demonstrate a similar enzyme activity in the gills of tilapia. Moreover, the present data provide evidence for a correlation between this enzyme activity and ATP-dependent Ca^{2+}-transport. The Ca^{2+}-transport process is homogenous and half-maximum activation occurred at intracellular Ca^{2+}-concentrations. The data provide the first biochemical support for active Ca^{2+}-transport in plasma membranes of teleost branchial epithelium. The magnitude of the calculated *in vitro* Ca^{2+}-transport capacity of the gills is consistent with an involvement of this mechanism in the uptake of Ca^{2+} from the water by the intact fish.

Membrane isolation and orientation

To determine high-affinity Ca^{2+}-ATPase activity in isolated plasma membranes by means of colorimetric methods, the availability of highly-purified plasma mem-

brane fractions is a prerequisite, for the maximum activity of this enzyme is low (approximately 5% of the Na^+/K^+-ATPase activity) and this activity has to be dissociated from background ATP-hydrolysis by membrane-bound, non-specific phosphatases (Flik *et al.*, 1984a). The two isolation procedures applied for tilapia gill plasma membranes, method I and II, yielded fractions with Na^+/K^+-ATPase activities that were 20% (V_{spec} = 30.9 µmol P_i/h.mg protein) and 50% (V_{spec} = 123.3 µmol P_i/h.mg protein) of the total ATPase activity, respectively. Ca^{2+}-ATPase activity was successfully determined only in membrane fractions obtained by method II. A major flaw of this method, however, is the low recovery of protein in the plasma membrane fraction (P_3'). This allowed only a limited number of individual determinations.

The isolation of membranes from tilapia branchial epithelium according to method I resulted in lower recoveries of SDH activity and Na^+/K^+-ATPase activity than was the case in eel gill membrane preparations (Flik *et al.*, 1983). Also, Na^+/K^+-ATPase specific activities were about 4-fold lower in the tilapia preparations than in the eel preparations. These differences may reflect species-specific differences, or may be attributed to differences in body size or water composition. However, the Na^+/K^+-ATPase specific activities in tilapia membranes isolated according to method I are similar to those reported for plasma membrane preparations of rat enterocytes (De Jonge *et al.*, 1981) or kidney cortex cells (Van Heeswijk *et al.*, 1984), preparations that were both shown to possess Ca^{2+}-transport activity.

Our results concerning the effects of EDTA and detergent treatment demonstrate that the presence of EDTA during isolation causes a leaky membrane preparation, whereas omission of this chelator during isolation yields at least partly a tight-vesicle preparation. Also, judged by the relative maximum activities of the Na^+/K^+-ATPase, omission of EDTA during isolation does not affect the composition of the membrane preparation. The resealing of membranes isolated in the absence of EDTA was further substantiated in substrate exclusion tests for acetylcholine esterase and GPDH activities. Average values for composition of the plasma membrane vesicle preparation were: 30% inside-out, 44% right-side-out and 26% leaky vesicles. These figures must be interpreted with care, however, for a 45% Na^+/K^+-ATPase accessibility of the same membrane preparation indicates that some permeation of ATP and ouabain occurred in a 10-min period (the incubation time for the Na^+/K^+-ATPase assay). This could imply that also the permeability of the membranes to Ca^{2+} increases during incubation. Thus, our calculated percentages of resealed vesicles probably are over-estimates when these values are used to assess membrane permeability to Ca^{2+}. Short incubation times, as used in our study, seem to

be a prerequisite for the determination of the Ca^{2+}-transport activity of plasma membrane vesicle preparations.

Ca^{2+}-stimulated ATPase activity

A high- and a low-affinity site were deduced for Ca^{2+}-stimulated ATPase activity, which corroborates our results on eel gill plasma membranes (Flik *et al.*, 1984b). Surprisingly, the same ratio for Na^+/K^+-ATPase to high-affinity Ca^{2+}-ATPase, namely 20.4 to 1, was observed in tilapia and eel gill plasma membranes. The affinity for Ca^{2+} of the low-affinity component excludes its involvement in the extrusion of Ca^{2+} from the cytosol, since, reportedly, in the latter compartment the Ca^{2+}-concentration is in general considerably lower than 14 µM (Wolf & Brostrom, 1979). No reports on free Ca^{2+}-levels in the cytosol of branchial epithelial cells are available in the literature, but the occurrence of high-affinity Ca^{2+}-ATPase and of calmodulin in branchial epithelium indicates that intracellular free Ca^{2+}-levels in this epithelium of 0.063 µM Ca^{2+} for the high-affinity component is within the range thay may be expected for an intracellularly stimulated enzyme. As pointed out earlier (Flik *et al.*, 1984a) such very high affinities for Ca^{2+} are an indication that the enzyme is calmodulin-dependent. In eel gill epithelium we have demonstrated that the high-affinity Ca^{2+}-ATPase activity is sensitive to calmodulin-antagonists (Flik *et al.*, 1984a). In the present study we have demonstrated the presence of calmodulin in Ca^{2+}-ATPase containing membranes of tilapia gills. However, whether the Ca^{2+}-ATPase in tilapia gills is also directly associated with calmodulin remains to be demonstrated. A close association of calmodulin with plasma membranes is indicated by the fact that our membranes were isolated in the presence of EDTA.

The determination of calmodulin in cytosol-fractions of branchial epithelium - a mixed cell population including mucus cells, chloride cells and respiratory cells - does not allow to specifically establish the cellular origin of calmodulin. We have shown that mucus contains calmodulin (Flik *et al.*, 1984c). However, mucus and cytosol-fractions of branchial epithelium purified in the same way, differ significantly in their calmodulin content: mucus contains 0.7 mg/g protein (Flik *et al.*, 1984c), cytosol contains 1.98 mg/g protein. Since mucocytes are almost completely filled up by mucus-containing secretory granules, the calmodulin determined in cytosol in the present study can not be derived from mucocytes only. Moreover, the finding that calmodulin is present in the Na^+/K^+-ATPase-enriched membrane fractions indicates that the chloride cells also contain an appreciable amount of calmodulin. In one experiment on seawater tilapia we

observed a three-fold higher calmodulin concentration in fractions of isolated chloride cells than in fractions of branchial epithelium as a whole (unpublished observation). In our membrane preparations high-affinity Ca^{2+}-ATPase activity is strictly associated with Na^+/K^+-ATPase activity, which latter enzyme activity is concentrated in the chloride cells of the gills (Shirai, 1972; Hootman & Philpott, 1979). It is reasonable to suggest then, that the high-affinity Ca^{2+}-ATPase is also concentrated in the chloride cells. The inference that calmodulin and high-affinity Ca^{2+}-ATPase activity are concentrated in the chloride cells of the gills supports the thesis by Payan *et al.* (1981) that these cells provide for virtually all of the branchial Ca^{2+}-transport.

The level of 0.165% calmodulin on H_0-protein basis makes the branchial epithelium a rich source of calmodulin comparable to e.g. mammalian testis (Wolf & Brostrom, 1979). This abundance of calmodulin in gills may be partially related to the important role of the latter structures in the uptake of Ca^{2+} from the water.

Ca^{2+}-transport in plasma membranes

Branchial influx of Ca^{2+} in freshwater fish is determined by transcellular Ca^{2+}-movement and, therefore, necessitates active transport (Flik *et al.*, 1984d). Our observation of ATP-dependent Ca^{2+}-transport in plasma membrane vesicles demonstrates directly the likeliest mechanism involved in this transepithelial flux. The high affinity for Ca^{2+} of this transporting process is consistent with an extrusion system that is stimulated by intracellular concentrations of Ca^{2+}. The maximum Ca^{2+}-transporting activity observed (580 nmol/h at 28°C) is approximately 4.5 times lower than the maximum transporting activity expected on the basis of calculated total high-affinity Ca^{2+}-ATPase activity in the branchial apparatus (2633 nmol/h). Since the Ca^{2+}-ATPase determination and the analysis of Ca^{2+}-transport required isolation of membranes by two different procedures, no conclusions concerning the ATP/Ca^{2+} stoichiometry of the process can be drawn. Yet, for a 20-g tilapia the Ca^{2+}-transporting capacity of the branchial apparatus (580 nmol/h), derived from *in vitro* experiments, compares well with its *in vivo* branchial Ca^{2+}-influx rate (560 nmol/h; Flik *et al.*, 1984d). Thus, it seems justified to ascribe physiological significance to the observed Ca^{2+}-transporting activity of our plasma membrane preparation.

Further characterization of the Ca^{2+}-transporting mechanism in fish gill epithelium is now being undertaken. In mammals the regulation of the levels of ionized calcium in the cytosol of ion-transporting epithelia may involve, in addition to transport Ca^{2+}-ATPase, a process of coupled Na^+/Ca^{2+}-exchange across

basolateral plasma membranes, energized by a sodium gradient over these membranes (Taylor & Windhager, 1979). The existence of Na^+/Ca^{2+}-exchange in fish gills and, next, the possible role of Na^+/Ca^{2+}-exchange in branchial Ca^{2+}-transport need to be considered.

REFERENCES

BORST PAUWELS, G.W.F.H. (1973) Two site-single carrier transport kinetics. J.theor.Biol. 40: 19-31.

DE JONGE, H.R., GHIJSEN, W.E.J.M. & VAN OS, C.H. (1981) Phosphorylated intermediates of Ca^{2+}-ATPase and alkaline phosphatase in plasma membranes from rat duodenal epithelium. Biochim.Biophys.Acta 647: 140-149.

FLIK, G., WENDELAAR BONGA, S.E. & FENWICK, J.C. (1983) Ca^{2+}-dependent phosphatase and ATPase activities in eel gill plasma membranes- I. Identification of Ca^{2+}-activated ATPase activities with non-specific phosphatase activities. Comp.Biochem.Physiol. 76B: 745-754.

FLIK, G., WENDELAAR BONGA, S.E. & FENWICK, J.C. (1984a) Ca^{2+}-dependent phosphatase and Ca^{2+}-dependent ATPase activities in eel gill plasma membranes- II. Evidence for transport high-affinity Ca^{2+}-ATPase. Comp.Biochem.Physiol. In press.

FLIK, G., WENDELAAR BONGA, S.E. & FENWICK, J.C. (1984b) Ca^{2+}-dependent phosphatase and Ca^{2+}-dependent ATPase activities in plasma mebranes of eel gill epithelium.- III Stimulation of high-affinity Ca^{2+}-ATPase activity during prolactin induced hypercalcemia in American eels. Comp.Biochem.Physiol. In press.

FLIK, G., VAN RIJS, J.H. & WENDELAAR BONGA, S.E. (1984c) Evidence for the presence of calmodulin in fish mucus. Eur.J.Biochem. 138: 651-654.

FLIK, G., FENWICK, J.C., KOLAR, Z., MAYER-GOSTAN, N. & WENDELAAR BONGA, S.E. (1984d) Whole body Ca^{2+}-flux rates in the freshwater cichlid teleost fish *Oreochromis mossambicus*, adapted to fresh water. Am.J.Physiol. Submitted.

GHIJSEN, W.E.J.M., DE JONG, M.D. & VAN OS, C.H. (1982) ATP-dependent calcium transport and its correlation with Ca^{2+}-ATPase activity in basolateral plasma membranes of rat duodenum. Biochim.Biophys.Acta 689: 327-336.

HEESWIJK, M.P.E. VAN, GEERTSEN, J.A.M. & VAN OS, C.H. (1984) Kinetic properties of the ATP-dependent Ca^{2+}-pump and the Na^+/Ca^{2+}-exchange system in basolateral membranes from rat kidney cortex. J.Membrane Biol. 79: 19-31.

HOOTMAN, S.R. & PHILPOTT, C.W. (1979) Ultracytochemical localization of Na^+, K^+-activated ATPase in chloride cells from the gills of a euryhaline teleost. Anat.Rec. 193: 99-130.

PAYAN, P., MAYER-GOSTAN, N. & PANG, P.K.T. (1981) Site of calcium uptake in the freshwater trout gill. J.exp.Zool. 216: 345-347.

SARKADI, B. (1980) Active calcium transport in human red cells. Biochim.Biophys. Acta 604: 159-190.

SHIRAI, N (1972) Electron-microscope localization of sodium ions and adenosine-triphosphatase in chloride cells of the Japanese eel, *Anguilla japonica*. J.of the Faculty of Science, University of Tokyo, Sec. IV, Vol. 12(3): 385-403.

STECK, T.L. & KANT, J.A. (1974) In: Methods in Enzymology 31A: 172-180. Academic Press, New York/London.

TAYLOR, A. & WINDHAGER, E.E. (1979) Possible role of cytosolic calcium and Na-Ca exchange in regulation of transepithelial sodium transport. Am.J.Physiol. 236(6): F505-F512.

TEO, T.S., WANG, T.H. & WANG, J.H. (1973) Purification and properties of the protein activator of bovine heart cyclic adenosine 3',5'-monophosphate phosphodiesterase. J.Biol.Chem. 248: 588-595.

WENDELAAR BONGA, S.E. & VAN DER MEIJ, J.C.A. (1980) The effect of ambient calcium osmolarity and calcium on prolactin cell activity and osmotic water permeability of the gills of the teleost *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. 43: 432-442.

WOLFF, D.J. & BROSTROM, C.O. (1979) Properties and functions of the calcium-dependent regulator protein. In: Advances in cyclic nucleotide research, Vol. 11 (Greengard, P. & Robison, G.A. eds) Raven Press New York: 27-88.

PART II

Ca^{2+}-EXCHANGE WITH THE WATER AND INTERNAL DISTRIBUTION OF CALCIUM IN A TELEOST FISH, *OREOCHROMIS MOSSAMBICUS*

Symbols (units) and definitions of symbols.

q_a (cpm;dpm)	Quantity of tracer in compartment a
Q_a (mol)	Quantity of tracee (the material traced) in compartment a
V_a (l)	Medium volume of compartment a
SA_a	Specific activity in compartment a; $SA_a = q_a/Q_a$
F_{ab} (mol/h)	Flux rate or flow into compartment a from compartment b
D^a	Dose of tracer administered to compartment a
T (h)	Time
W (g)	Wet weight

Subscripts:

f	Fish
w	Water
p	Plasma
0	Zero time

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WHOLE-BODY CALCIUM FLUX RATES IN THE CICHLID TELEOST FISH *OREOCHROMIS MOSSAMBICUS*, ADAPTED TO FRESH WATER

ABSTRACT

Radiotracer techniques were used to measure influx and efflux rates of calcium in fresh water adapted *Oreochromis mossambicus*. The influx rate of calcium is related to body weight (W) as $F_{fw} = 50W^{0.805}$ nmol/h Ca^{2+}. For a 20-g fish the calculated influx rate was 558 nmol/h Ca^{2+} and this was attributed largely to extra-intestinal uptake since the drinking rate was only 28 µl/h, which corresponds to an intake of 22.4 nmol/h Ca^{2+}. The Ca^{2+}-efflux rate was calculated using the rate of appearance of radiotracer in the ambient water and the specific activity of plasma calcium. Tracer efflux rates were constant over 6-8 hours and this was taken to indicate that there was no substantial loss of tracer in either the urine or the faeces as this would have resulted in random bursts of tracer loss. Efflux rates then primarily represent integumentary and, presumably, branchial efflux rates. The efflux rate of Ca^{2+} is related to body weight as $F_{wf} = 30W^{0.563}$ nmol/h Ca^{2+}, which means an efflux rate of 162 nmol/h Ca^{2+} for a 20-g fish. Taking these two flux rates into account, the calculated net whole body Ca^{2+}-influx was 396 nmol/h for a 20-g fish and this proves that the ambient water is an important source of calcium.

INTRODUCTION

Freshwater teleostean fish such as *Oreochromis mossambicus* (hereafter called tilapia) maintain their plasma calcium levels within narrow limits (Fenwick & Wendelaar Bonga, 1982). As is the case for most teleosts, this species continues to grow under natural conditions and must, therefore, continually increase its amounts of whole-body calcium. To satisfy this constant requirement for calcium uptake and to compensate for calcium losses caused by outward diffusion across the integument and via the production of urine and faeces, freshwater fish actively accumulate calcium from both food and water. However, direct absorption of Ca^{2+} from the water by the gills is believed to be the predominant route for

Ca^{2+}-uptake in freshwater fish (Berg, 1968, 1970; Ichii & Mugiya, 1983). This report deals with the significance of the extra-intestinal calcium uptake.

The understanding of calcium fluxes in teleost fish is limited by a paucity of studies. Furthermore, most studies concerned with calcium handling by fish involved seawater species only. Since fresh water and sea water conditions are very different with respect to calcium, one should be cautious in comparing studies on calcium metabolism in seawater fish with those concerning freshwater fish. Sea water contains very high levels of calcium and in such an environment fish are most likely forced to compensate for excessive influx of calcium by secreting calcium. Conversely, active uptake of calcium is a requisite for survival and growth in fresh waters, where Ca^{2+}-concentrations are often much lower than those of the body fluids. Moreover, the osmolarity and the concentrations of ions other than Ca^{2+} are very much different in fresh water and in sea water. Ambient concentrations of one particular ion may well affect the fish's physiological activity with respect to another ion. The high levels of Mg^{2+} in sea water, for example, influence the Ca-physiology of fish adapted to Ca^{2+}-deficient sea water (Wendelaar Bonga *et al.*, 1983).

This study forms part of our investigations on environmental impact and endocrine control of Ca-metabolism in tilapia and deals with whole-body influx and efflux rates of Ca^{2+} in tilapia adapted to artificial fresh water containing 0.8 mM $CaCl_2$. Whole-body influx of Ca^{2+} was studied by determining the movement of $^{47}Ca^{2+}$ into intact live fish, using a whole-body counter. Tracer retention was studied after fish were given an intraperitoneal injection of $^{47}Ca^{2+}$. Whole-body efflux rates of Ca^{2+} were estimated by determining $^{45}Ca^{2+}$-loss from the fish four days after tracer injection.

While Ca^{2+}-influx data are available for a limited number of freshwater species (Berg, 1968, 1970; Fleming, 1973; Fleming *et al.*, 1973; Pang *et al.*, 1980; Ichii & Mugiya, 1983; Mayer-Gostan *et al.*, 1983; Höbe *et al.*, 1984), to our knowledge this is the first report on Ca^{2+}-efflux rates for freshwater fish. Such efflux rates are essential to assess net uptake of Ca^{2+} from the water and to evaluate the role of the ambient water as a Ca-source for fish. The present data enabled us to estimate the contribution of calcium taken up from the water to the calcium balance of the fish.

MATERIALS AND METHODS

Male tilapia, *Oreochromis mossambicus* (formerly called *Sarotherodon mossambicus*),

were obtained from laboratory stock, kept in Nijmegen tap water. The conditions were the same as those described earlier (Wendelaar Bonga & Van der Meij, 1980), but the temperature was 28 ± 1°C.

Fish used for radiotracer studies were transferred into 100-l aquaria containing artificial fresh water made up from demineralized water and containing (mM): NaCl (3.8), KCl (0.06), $MgSo_4$ (0.2) and $CaCl_2$ (0.8). The pH was adjusted with $NaHCO_3$ to 7.4 ± 0.2 and the osmolarity was 8-10 mOsmol/l.

Although the composition of the artificial fresh water is essentially the same as the composition of the Nijmegen tap water, artificial fresh water was preferred for radiotracer studies in order to guarantee constant concentrations of Ca^{2+} and of other ions. During the experiments the pH and Ca concentration in water was monitored and adjusted if necessary. Nitrogen wastes were kept in all cases below 2 µM NH_4^+.

Calcium determination

Total body Ca was determined by digesting fish in concentrated HNO_3. Blood was collected from the caudal blood vessels and plasma samples were prepared as described previously (Wendelaar Bonga & Van der Meij, 1980). Total Ca of water, HNO_3-digests and plasma was analyzed by means of atomic absorption spectrophotometry, using $LaCl_3$ (20 mM) as diluent. To avoid contamination of the atomic absorption unit, ^{45}Ca-containing samples were determined with a commercial Ca-kit (Sigma).

Radiotracer techniques

In order to follow the Ca^{2+}-transport, radiotracers $^{45}Ca^{2+}$ and $^{47}Ca^{2+}$ were used. Both were purchased (Amersham International plc, England) as $CaCl_2$ in aqueous solution. Specific activities were 9.25-37.5 GBq/mol Ca and > 0.74 GBq/mol Ca for ^{45}Ca and ^{47}Ca, respectively. ^{45}Ca decays (half-life = 164 days) by β^--emission (E_{max} = 0.252 MeV) into stable ^{45}Sc. Its activity was measured by liquid scintillation counting (LKB Rackbeta LSA, equipped with a dpm-program) of samples prepared by mixing 1 ml of a ^{45}Ca-containing aqueous solution with 4 ml counting solution (Aqualuma, Lumac). ^{47}Ca decays (half-life = 4.54 days) by β^--emission followed by the emission of γ-rays (E = 0.49 (5%), 0.815 (5%) and 1.308 (74%) MeV) into ^{47}Sc, which also decays (half-life = 3.40 days) by β^--emission followed by the emission of γ-rays (E = 0.160 (73%) MeV). The ^{47}Ca activity in liquid samples and in whole fish was measured by a well-type, 3x3" NaI (Tl) scintillation detector equipped with an appropriate counter (gamma-ray

spectrometer: Elscint or LKB Ultrogramma II) set to measure 1.308 MeV photopeak of ^{47}Ca only. In this setting the contribution of ^{47}Sc-emission was completely excluded.

The counting efficiency for the 1.308 MeV radiation for 1 ml sample measured in the well amounted to about 15% (100 x cps/ 0.74 x Bq). A linear decrease of the counting rate (4% and 6.5% decrease per ml for the forecalled detectors, respectively) was observed with increasing sample volume. As a routine for all but the 1-ml samples, appropriate corrections were made, which led to an apparent counting rate corresponding to 1 ml sample volume.

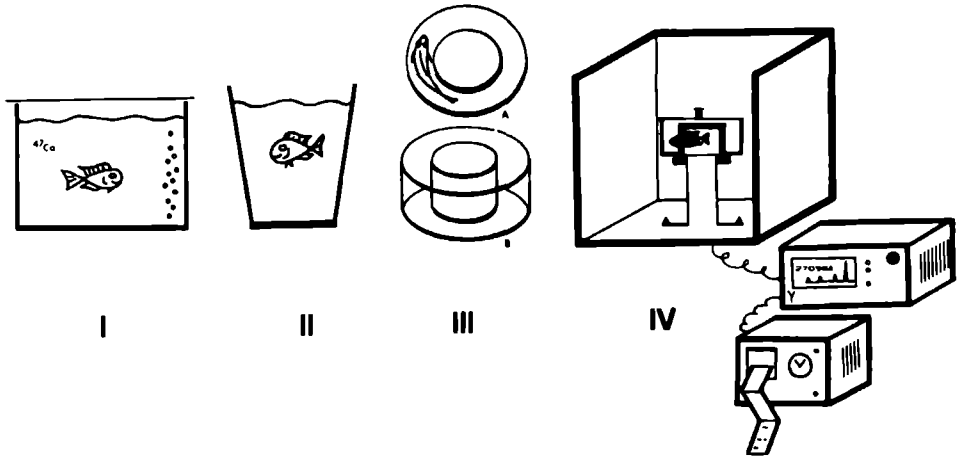
Whole-body ^{47}Ca activity of live fish was measured using a perspex container with a ring-shaped compartment (1 l), mounted concentrically around a well-type scintillation crystal (Fig. 1). In order to determine the counting efficiency for 1.308 MeV γ-rays for ^{47}Ca in the fish when measured alive relative to the counting efficiency for samples measured in the well of the scintillation crystals, the following procedure was applied. A ^{47}Ca-containing fish was rinsed (1 min) with tracer-free water to remove adsorbed tracer and the fish was counted in the perspex container. Then the fish was killed and quickly dissolved in 12 M KOH at 60°C and the bones were destroyed mechanically. The resulting digest was divided into 5-ml portions, that were counted in the well of the scintillation crystal. From the sum of the counting rates of all 5-ml portions and the counting rate obtained in the perspex container for the whole live fish the relative counting efficiency was calculated (100x counting rate fish/counting rate in the well). For fish of 10-30 g values of 19.0 ± 1.3% (n=5) were obtained. The counting efficiency for intact fish came to 2.7% in this set-up (100x cps/0.74 x Bq).

Animals were always counted after a 1-min rinse in tracer-free water to remove tracer adsorbed to the body surface. After being counted, the fish were removed from the container and then the water was counted for background activity. Counts collected for live fish surpassed at least 10 times background count rates. During transfers fish were handled with wet rubber gloves to prevent any skin damage that could influence the ion fluxes (Höbe *et al.*, 1984). Amounts of fish during the influx studies and tracer retention studies never exceeded 15 g fish per liter water.

Ca^{2+}-influx

For Ca^{2+}-influx studies all-glass aquaria or perspex containers contained 12 l or 1 l of well-aerated water, respectively. At the start of the influx experiments (2-8 h after addition of tracer to the water) fish were transferred to the exposure systems; they were not fed during influx experiments which lasted up to 3 h.

Figure 1. Uptake of $^{47}Ca^{2+}$ in live tilapia. Fish exposed to $^{47}Ca^{2+}$ in the water take up $^{47}Ca^{2+}$ in the body (I). At fixed time intervals fish are caught and transferred to tracer-free water to rinse off tracer adsorbed to the body surface (II). Next, the fish are transferred to a ring-shaped container with 1 l of water (III, A: top view; B: side view). This container fits around a scintillation crystal and is mounted in a lead chamber (IV, cover and front removed). The scintillation crystal detects the γ-emission in the fish. The gamma-ray spectrometer connected to the scintillation crystal, and equipped with a printer/clock allows analysis and registration of specific emissions of the ^{47}Ca. During the measurements the fish are free-swimming. Fish are handled with wet rubber gloves throughout transfers to prevent skin damage.



Drinking rates

Drinking rates were determined on the basis of gut ^{47}Ca contents as reported by Pang *et al.* (1980). Immediately after three hours exposure to tracer, the abdominal cavity was opened and the intestinal tract, minus the liver and the gallbladder, was assessed for ^{47}Ca radioactivity. Good care was taken to include the total intestinal contents. The volume of water consumed was calculated as cps per gut/cps per ml water. Drinking rates were expressed as µl/h water or nmol/h Ca^{2+}.

Ca^{2+}-efflux

First, whole-body tracer retention was studied in fish intraperitoneally injected with $^{47}Ca^{2+}$ (4.44×10^7 Bq/g). Fish were kept in all-glass aquaria with 40 l water; the water was circulated through charcoal filters which were previously equilibrated to the water. Filters and solutions were renewed if necessary to keep tracer activity at background levels in order to prevent tracer backflux. During tracer-retention experiments fish were normally fed. Secondly, fish were given a dose of $^{45}Ca^{2+}$ (D^f) and the plasma radioactivity was measured from 0.5 - 79 h after injection. Plasma radioactivity (q_p) was expressed as $q_p/V_p.D^f$. To determine Ca^{2+}-efflux rates fish were injected intraperitoneally with $^{45}Ca^{2+}$ (2.88×10^8 Bq/g) and were held and fed for three days. Then feeding was discontinued and the fish were starved for one day. Next, they were caught, the urinary bladder was emptied by gently pressing the posterior abdominal wall, and the fish were transferred to perspex containers with 0.5 - 1 l aerated water for 6 - 8 h. During this period and at the end of the experiment water samples were taken and their radioactivity determined.

Calculations

The influx rates of Ca^{2+} (F_{fw}: flux to fish from water) were calculated from the time-curves for the tracer content of the fish (q_f) normalized to the tracer content of the water at zero time (q_{w0}). The instantaneous initial upslope of these curves at zero time, $d(q_f/q_{w0})/dt$, is equal to F_{fw}/Q_w with Q_w being the amount of calcium in the water (Shipley & Clark, 1972; p194). Because of the inaccuracies in defining the early portion of the curve via observed points, a slope obtained by the least-squares-fitting of a line through the data points for up to three hours was used instead of the actual slope at zero time.

The efflux rates of Ca^{2+}(F_{wf}: flux to water from fish) were calculated from the time-curves for the tracer content of the water (q_w), which one may normalize to the tracer content of the blood plasma of the fish at zero time (q_{p0}). Zero time is the moment of the immersion into tracer-free water of fish previously injected with tracer and kept for three or four days in tracer-free water. The slope of these curves at zero time, $d(q_w/q_{p0})/dt$, is equal to F_{wp}/Q_p, Q_p being the amount of calcium in the plasma of the fish. The ratio q_{p0}/Q_p is equal to the specific activity of calcium in the plasma at zero time (SA_{p0}) and hence $F_{wf} = (dq_w/dt)/SA_{p0}$. Since SA_{p0} is not accessible for direct determination, SA_p at the end of the experiments was used instead (SA_p was shown not to decrease significantly over a 6-8 h period 4 days after tracer injection). Here also not

the actual slopes at zero time were used but the slopes obtained by least-squares-fitting of a line trhough the data points for up to 8 hours.

Statistics and notations

Student's t-test for unpaired observations or the Kruskal-Wallis one-way analysis of variance by ranks were applied to assess statistical significance of differences of mean values. Linear regression analysis was based on the least-squares-method. The symbols, definitions of symbols and units used were taken from Brownell *et al.* (1968) and Shipley & Clark (1972).

RESULTS

In freshwater male tilapia ranging in body weight from 4-93 g, the calcium content of the body amounts to 316.3 ± 2.3 µmol/g. A full-logarithmic plot of the results (Fig. 2) yields the power function for the total fish calcium pool: $Q_f = 357.5W^{0.965}$ µmol. The calculated slope of the regression line (0.965) approximates unity, which means that the size of the total body calcium pool is directly related to body weight.

Ca^{2+}-influx and drinking rates

Intact fish accumulated ^{47}Ca at a constant rate for at least 3 hours (Fig. 3). Drinking rates determined on the basis of intestinal ^{47}Ca-contents were 28.0 ± 14.2 µl/h for a 20-g fish, i.e. an intake of 22.4 ± 12.2 nmol/h Ca. The radioactivity present in the gut after three hours as a fraction of the estimated total body radioactivity averaged 1.26% and never exceeded 2.6%. Thus, although the slope of the tracer uptake curve reflects both the entry through the integument and the gut contents (which latter in fact belong to the external compartment), whole-body influx of calcium can be calculated from the slope of a 3-h tracer uptake curve and the specific activity of ^{47}Ca in the water. Since the fish size varied from 9.8 to 28 g in this group, we could establish a relation between influx rates and body weight. A positive correlation was observed between body weight and Ca^{2+}-influx. Rates of Ca^{2+}-influx ranged from 195 nmol/h (W = 9.8 g) to 1065 nmol/h (W = 28 g)(Fig. 4). In this weight range Ca^{2+}-influx rate is related to body weight as $F_{fw} = 50W^{0.805}$ nmol/h. For a 20-g fish F_{fw} comes to 558 nmol/h Ca^{2+}.

Figure 2. Full logarithmic plot of the relationship between body weight in grams (W/g) and whole body calcium content in µmol ($Q_f/10^{-6}$ mol). A highly significant correlation was observed between log(W/g) and log($Q_f/10^{-6}$ mol) ($r_0 = 0.998$, $P < 0.001$, n=25). Total body Ca for freshwater male tilapia can be described by $Q_f = 357.5W^{0.965}$ µmol Ca.

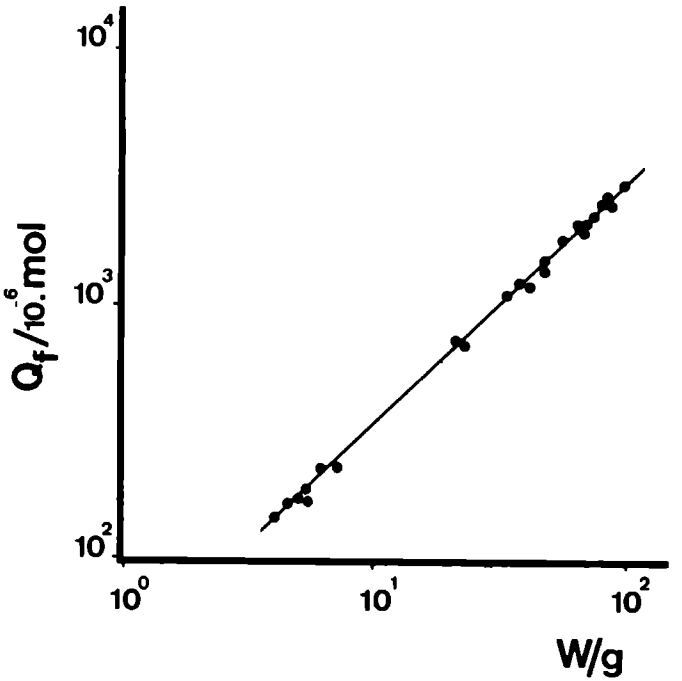


Figure 3. Accumulation of $^{47}Ca^{2+}$ (q_f/q_{w0}) in an individual fish expressed as a fraction of the total radioactivity in the water at zero time. The Ca^{2+}-influx rate is calculated from the slopes of such linear tracer uptake curves and the water total Ca pool. The calculated Ca^{2+}-influx rate for this fish (W=15.1 g) was 880 nmol/h.

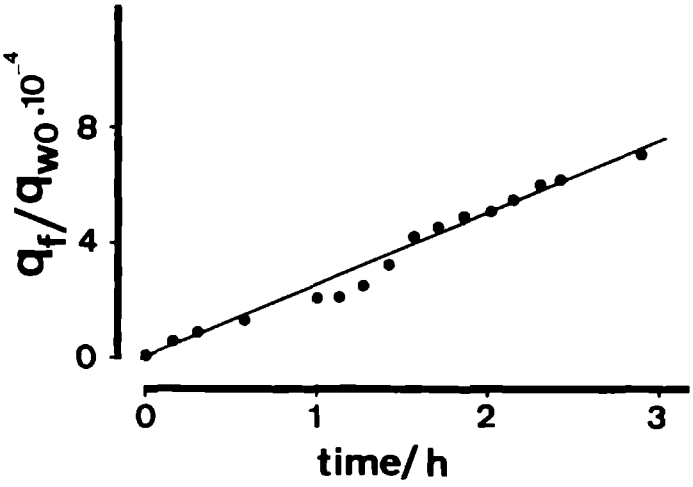
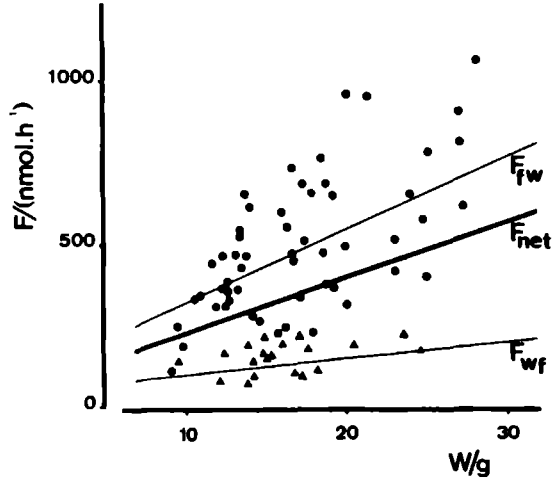


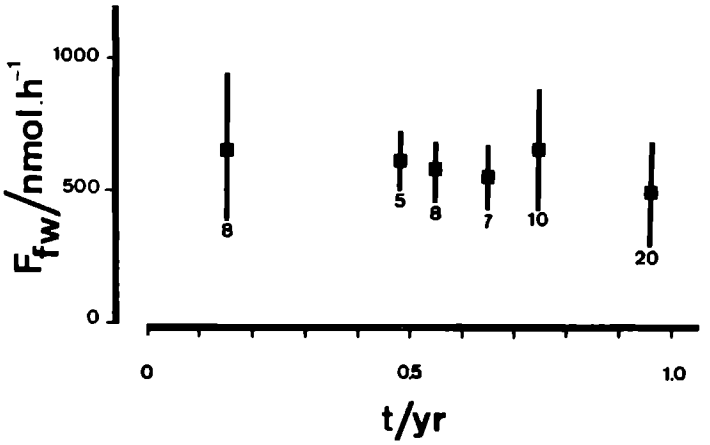
Figure 4. Relationships between whole-body Ca^{2+}-flux rates and body weight. For fish in the range of 9-28 g, a significant positive correlation (r_0=0.540, $P < 0.001$, n=58) was observed between Ca^{2+}-influx rate (F_{fw}) and body weight. Ca^{2+}-influx rates were satisfactorily described by $F_{fw} = 50W^{0.805}$ nmol/h.

For fish in the range of 9.8-24 g, Ca^{2+}-efflux rates were positively correlated with body weight (r_0=0.443, $P < 0.05$, n=21). Ca^{2+}-efflux rates were satisfactorily described by $F_{wf} = 30W^{0.563}$ nmol/h. Thick line represents net Ca^{2+}-influx rates, calculated as the difference between F_{fw} and F_{wf}.



To assess whether Ca^{2+}-influx in our fish underwent fluctuation throughout the year, all influx rates of individual fish ($F_{fw}(W)$) presented in Fig. 4 were converted to values for a 20-g fish, according to : $F_{fw}(20) = F_{fw}(W) \times (20/W)^{0.805}$. These values were subsequently pooled in groups per month of experimentation. These data (Fig. 5) showed that Ca^{2+}-influx rates in our freshwater male tilapia did not undergo statistically significant ($P > 0.20$) seasonal fluctuation.

Figure 5. Ca^{2+}-influx rates throughout the year. Individual Ca^{2+}-influx rates ($F_{fw}(W)$) were converted to influx rates for a 20-g tilapia, according to $F_{fw}(20 = F_{fw}(W) \times (20/W)^{0.805}$. These values were pooled per month of experimentation. No significant differences among the groups were observed (Kruskal-Wallis, one-way analysis of variance by ranks, $P > 0.20$).

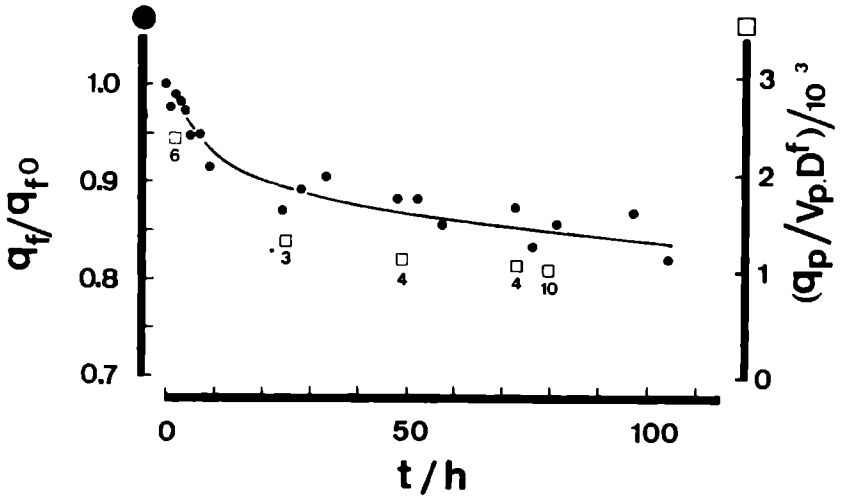


Ca^{2+}-efflux rates

For the determination of efflux rates plasma Ca-tracer specific activity should preferably be constant during the experiment. Fig. 6 shows that in live tilapia the first 24 h after tracer injection were marked by a rapid decrease in whole-

body tracer content. This was followed by a small, apparently linear decrease which lasted for at least a further 60 h. In a separate experiment, using ^{45}Ca, plasma-tracer activity ran concurrently with whole-body tracer content. Plasma tracer content ($q_p/V_p.D^f$) at 72 h and 79 h - the time at which the efflux experiments were done - were (1.079 ± 0.114) x 10^{-3} ml^{-1} (n=4) and (1.058 ± 0.327) x 10^{-3} ml^{-1} (n=10), respectively, and these values did not differ in a statistically significant way ($P > 0.15$).

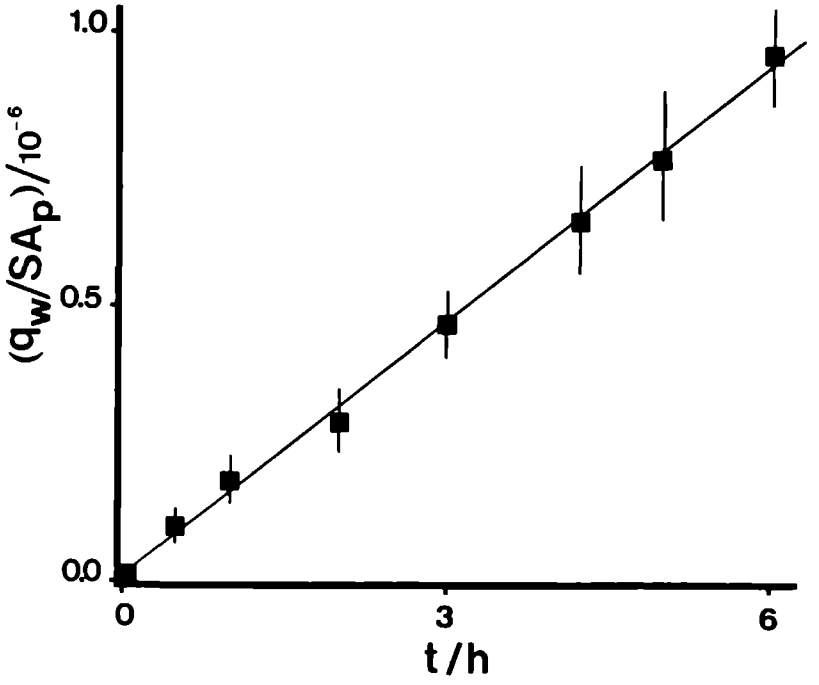
Figure 6. Whole-body tracer-retention curve for tilapia injected intraperitoneally with $^{47}Ca^{2+}$. Tracer retained in the body (q_f) is expressed as a fraction of the tracer in the fish at zero (injection) time (q_f/q_{f0}). Mean values of 7 fish are shown (● ——— ●). Plasma tracer content (q_p/V_p) expressed as a fraction of the dose of tracer injected (D^f) decreases concurrently with whole-body tracer content (▭). Numbers indicate the number of fish per group.



From these observations we conclude that between 72 h and 100 h after injection of the tracer, its loss from the body is slow and linear and that the plasma Ca-tracer specific activity will not change significantly over a 6 to 8 h period. For 10 fish killed 79 h after tracer injection, plasma total Ca amounted to 2.85 ± 0.13 mM; such values are in line with previously reported plasma Ca levels in this species (Wendelaar Bonga & Van der Meij, 1980).

Tracer accumulation rate in the water proved to be constant over a 6-8 h period (Fig. 7).

Figure 7. Tracer appearance in the water upon immersion of $^{45}Ca^{2+}$-injected fish in tracer-free water. Water tracer content (q_w) is expressed as the fraction of the plasma ^{45}Ca specific activity of the fish at the end of the experiment (SA_p). Mean values for 10 fish ± S.E. are given. Regression line : $r_0 = 0.999$, $P < 0.001$.



On the basis of the data presented in Fig. 5, and the accumulation rates of tracer in the water, Ca^{2+}-efflux rates (Fig. 4) were calculated. Individual Ca^{2+}-efflux rates ranged from 74 nmol/h (in a fish weighing 13.8 g) to 229 nmol/h (in a fish weighing 23.6 g). In the body weight trajectory studied, efflux rates of Ca^{2+} were related to body weight as $F_{wf} = 30W^{0.563}$ nmol/h. For a 20-g fish F_{wf} comes to 162 nmol/h Ca^{2+}. According to the conservation equation $F_{net} = F_{fw} - F_{wf}$, a net influx rate of 392 nmol/h Ca^{2+} was calculated for a 20-g male tilapia in fresh water.

DISCUSSION

Ca^{2+}-influx

To estimate Ca^{2+}-uptake from the water in tilapia we determined bidirectional Ca^{2+}-fluxes between fish and water. An influx rate of 558 nmol/h Ca^{2+} for a 20-g tilapia was calculated, which is of the same order as influx rates reported for other species, e.g. *Fundulus kansae* (27 nmol/h/g wet weight; Fleming *et al.*, 1973), *Carassius auratus* (16.3 nmol/h/g fish; Berg, 1968) and *Fundulus heteroclitus* (32.5 nmol/h/g fish; Pang *et al.*, 1980), but significantly lower than the Ca^{2+}-influx rate for *Carassius auratus* (149 nmol/h/g fish) reported by Ichii & Mugiya (1983).

Ca^{2+} from the water enters the fish via the gut by drinking water and via the integument, of which the gills form a major part (Hughes, 1972). The drinking rate of 28 µl/h water for a 20-g fish as found in this study is about half that of the values reported by Potts *et al.* (1976) for the same species in fresh water (0.26% of the body weight per hour) but we do not consider this difference physiologically significant. Reported values for other species are: for *Carassius auratus* 51 µl/h/100 g (Motais *et al.*, 1969), for *Anguilla anguilla* 135 µl/h/100 g (Maetz & Skadhauge, 1968), for freshwater *Platichthys flesus* 37 µl/h/100 g (Motais *et al.*, 1969) and for *Salmo trutta* 45.5 µl/h/100 g (Oduleye, 1975). If we assume that all of the Ca^{2+} entering tilapia by drinking is absorbed from the gut, the intestinal influx would amount to 22.4 nmol/h Ca^{2+} for a 20-g fish, which is equivalent to 3.9% of the total body Ca^{2+}-influx in freshwater adapted fish. Admittedly, objection can be made to the calculation of drinking rates from Ca-tracers present in the contents of the intestine. In tilapia bidirectional transmural Ca^{2+}-fluxes in the gut occur (Flik *et al.*, 1982). Consequently, bidirectional tracer fluxes will occur in the gut. If, for example, ^{47}Ca, after having been taken up by the gills, exchanged with gut Ca-contents, the drinking rate

mentioned above for tilapia is an overestimate. The gut radioactivity was smaller than the standard error of the mean total body tracer accumulated in 3 h. For the time being then, we consider the total body radioactivity to approximate the extraintestinal influx of $^{47}Ca^{2+}$. We further consider the gills as the main site of Ca^{2+}-entry. Mashiko & Jozuka (1964) suggested that in freshwater *Carassius auratus* the integument covering the body and especially that of the fins, is an additional site for Ca^{2+}-exchange. Unfortunately, the relative contributions of Ca^{2+}-exchange through skin and fins have so far not properly been quantified, i.e. in terms of net Ca^{2+}-fluxes. It may be assumed that the relative contribution of branchial and epidermal Ca^{2+}-exchange is at most proportional to the surface of these parts. Estimates for the gill surface area in most fish range from 70-95% of the total body surface (Hughes, 1972; Muir & Hughes, 1969). It is more likely however, that the gills contribute significantly more to the Ca^{2+}-uptake than their relative surface area suggests. In the first place, the gills are substantially better vascularized than the skin. Further, the gills contain most of the chloride cells of the integument, and there is evidence that these cells are specialized for the uptake of Ca^{2+} (Payan *et al.*, 1981). We have shown that in *Anguilla rostrata* the high-affinity Ca^{2+}-ATPase which we consider the enzymic expression for the branchial Ca^{2+}-pump is most likely located in the chloride cells (Flik *et al.*, 1984). Similar, unpublished, observations were made on tilapia. No such enzyme activity could be detected in the skin. These observations support the idea that the gills are the main site for active Ca^{2+}-uptake, and that the whole-body Ca^{2+}-influx essentially reflects branchial influx.

Our Ca^{2+}-influx estimates are very similar to those reported for isolated head preparations of freshwater trout (Payan *et al.*, 1981) but not to those reported on the gill arch preparation of European eel by Milet *et al.* (1979). The observed relationship between body weight and Ca^{2+}-influx rate ($F_{fw} = 50W^{0.805}$ nmol/h) is strikingly similar to the relationship between body weight and water exchange reported for freshwater tilapia by Potts *et al.* (1967). A similar function with a power of 0.85 was reported for the relationship between body weight and oxygen-consumption in fish (Winberg, 1956). The considerable spread in individual values for whole-body Ca^{2+}-influx rate may result from individual differences in general metabolic activity or Ca-status of the fish. Alternatively, one might assume the existence of a direct relationship between gill surface and Ca^{2+}-influx rates. As the gill surface area is reported to vary considerably for individuals of the same species (Hughes, 1972), this could explain the observed individual variation.

In our study we did not observe seasonal variation in Ca^{2+}-influx rates.

The fact that the fish were kept under constant conditions throughout the year and that only males were used in the present study may, however, have eliminated any season- or sex- related variations. Fleming *et al.* (1979) reported seasonal variations in Ca^{2+}-influx in *Fundulus kansae*, which they tentatively related to changes in the endocrine status of the fish. Seasonal variations in responses to adrenalin have been reported for *Salmo gairdneri* (Paert *et al.*, 1982) and such variations influence branchial Ca^{2+}-uptake (Payan *et al.*, 1981).

Ca^{2+}-efflux

Calculations of whole-body Ca^{2+}-efflux rates were based on plasma total ^{45}Ca specific activity. We assumed that no differences in tracer specific activity existed between protein-bound calcium fractions and the dialyzable (complexed and ionic) calcium fractions, neither in the blood plasma nor in the extracellular fluids. A second assumption is, that plasma Ca-tracer specific activities did not change significantly during the efflux experiment. This was justified by the constancy of plasma tracer levels during the period of experimentation (i.e. on the fourth day after tracer injection). A double constraint applies to the calculation of Ca^{2+}-efflux rates in our set-up. First, the duration of the experiment must be short in comparison with the time required for isotope equilibration between fish and water. Secondly, during the experiment the specific activity in the compartment from which the tracer leaves should be constant. Apparently, both conditions were fulfilled, as was indicated by the constancy of efflux rate values during the experimental period (Fig. 7).

The question arises then to what extent whole-body efflux rates of Ca^{2+} reflect branchial efflux rates. In addition to branchial Ca^{2+}-efflux, urinary and intestinal excretion of Ca^{2+} take place. The tracer accumulation in the water for individual fish was linear over a 6-8 h period and this was taken as evidence that no substantial urinary or faecal Ca^{2+}-excretion occurred, since such periodical phenomena would have resulted in a fluctuating efflux. Efflux via the integument may be considered to occur by passive diffusion only. Therefore, and because the skin is much thicker and less vascularized than the gills (Wendelaar Bonga & Meis, 1981), Ca^{2+}-efflux through the skin is probably relatively small. To our knowledge, no reports on whole-body Ca^{2+}-efflux rates are available in the literature. An efflux rate of 229 nmol/h Ca^{2+} in a 20-g tilapia, however, compares well with a branchial efflux rate of 6.93 µmol/h/kg fish reported by Milet *et al.* (1979) for isolated gill arch preparations of European eel.

Net Ca^{2+}-flux

A freshwater adapted tilapia of 20 g has a net uptake rate of Ca^{2+} from the water of about 400 nmol/h. This uptake rate will allow a freshwater tilapia of 20 g to grow by 1 g, which is equivalent to the accumulation of 310 µmol Ca in the body, in 33 days. This value is commensurate with growth rate under our laboratory conditions. An experiment whereby tilapia were fed a calcium-deficient diet did not significantly affect their growth rate (unpublished results). Apparently, tilapia have an efficient system to extract Ca^{2+} from the water. Berg (1968, 1970) has shown for *Carassius auratus* that intestinal Ca-absorption and branchial Ca^{2+}-influx are inversely related. We have preliminary evidence that in tilapia the Ca^{2+}-uptake from the water can be hormonally stimulated (Wendelaar Bonga & Flik, 1982). It would be interesting to investigate whether the process of adjusting branchial Ca^{2+}-uptake to the dietary supply in tilapia is mediated by hormones.

REFERENCES

BERG, A. (1968) Studies on the metabolism of calcium and strontium in freshwater fish. I- Relative contribution of direct and intestinal absorption. Mem.Ist. Ital.Idrobiol. 23: 161-196.

BERG, A. (1970) Studies on the metabolism of calcium and strontium in freshwater fish. II- Relative contribution of direct and intestinal absorption in growth conditions. Mem.Ist.Ital.Idrobiol. 26: 241-255.

BROWNELL, G.L., BERMAN, M. & ROBERTSON, J.S. (1968) Nomenclature for tracer kinetics. Int.J. of Applied Radiation and Isotopes 19: 249-262.

CHANNA, A. (1981) A comparative histochemical study of calcium absorption in three freshwater teleosts. Acta Histochem.Cytochem. 14(6): 563-570.

FENWICK, J.C. & WENDELAAR BONGA, S.E. (1982) Hormones and osmoregulation: endocrine involvement in calcium regulation in teleosts. In: Exogenous and endogenous influences on metabolic and neural control, Vol.I., edited by Addink, A.D.F. & Spronk, N. Osford: Pergamon Press, p 339-350.

FLEMING, W.R. (1973) Electrolyte metabolism of teleosts including calcified tissues. In: Chemical Zoology Vol.8 edited by Florkin, M. & Scheer, B.T. New York: Academic press, p 471-508.

FLEMING, W.R., BREHE, J. & HANSON, R. (1973) Some complicating factors in the study of the calcium metabolism of teleosts. Amer.Zool. 13: 793-797.

FLIK, G., REIJNTJENS, F.M.J., STIKKELBROECK, J. & FENWICK, J.C. (1982) 1,25-Vit D_3 and calcium transport in the gut of tilapia *(Sarotherodon mossambicus)*. J.Endocrinol. 94: 40P.

FLIK, G., WENDELAAR BONGA, S.E. & FENWICK, J.C. (1983) Ca^{2+}-dependent phosphatase and ATPase activities in eel gill plasma membranes I- Identification of Ca^{2+}-activated ATPase activities with non-specific phosphatase activities. Comp.Biochem.Physiol. 76B(4): 745-754.

FLIK, G., WENDELAAR BONGA, S.E. & FENWICK, J.C. (1984) Ca^{2+}-dependent phosphatase and Ca^{2+}-dependent ATPase activities in eel gill plasma membranes II- Evidence for transport high-affinity Ca^{2+}-ATPase. Comp.Biochem.Physiol.: In press.

HOBE, H., WOOD, C.M. & McMAHON, B.R. (1984) Mechanisms of acid-base and ionoregulation in white suckers *(Catostomus commersoni)* in natural soft water. J.Comp.Physiol. B 154: 35-46.
HUGHES, G.M. (1972) Morphometrics of fish gills. Respiration Physiology 14: 1-25.
ICHII, T. & MUGIYA, Y. (1983) Effects of a dietary deficiency in calcium on growth and calcium uptake from the aquatic environment in the goldfish *Carassius auratus*. Comp.Biochem.Physiol. 74A(2): 259-262.
MAETZ, J. & SKADHAUGE, E. (1968) Drinking rates and ionic turnover in relation to external salinities in the eel. Nature, Lond. 217: 371-3.
MASHIKO, K. & JOZUKA, K. (1964) Absorption and excretion of calcium by teleost fishes with special reference to routes followed. Ann.Zool.Jap. 37(1): 41-50.
MAYER GOSTAN, N., BORNANCIN, M., DeRENZIS, G., NAHON, R., YEE, J.A., SHEW, R.L. & PANG, P.K.T. (1983) Extra-intestinal calcium uptake in the killifish *Fundulus heteroclitus*. J. of exp. Zool. 227(3): 229-339.
MILET, C., PEIGNOUX-DEVILLE, J. & MARTELLY, E. (1979) Gill calcium fluxes in the eel, *Anguilla anguilla (L)*. Effects of Stannius corpuscles and Ultimobranchial Body. Comp.Biochem.Physiol. 63A: 63-70.
MOTAIS, R., ISAIA, J., RANKIN, J.C. & MAETZ, J. (1969) Adaptive changes of the water permeability of the teleostean gill epithelium in relation to external salinity. J.exp. Biol. 51: 529-546.
MUIR, B.S. & HUGHES, G.M. (1969) Gill dimensions for three species of tunny. J.exp. Biol. 51: 271-285.
ODULEYE, S.O. (1975) The effects of calcium on water balance of the brown trout *Salmo trutta*. J.exp. Biol. 63: 343-356.
PANG, P.K.T., GRIFFITH, R.W., MAETZ, J. & PIC, P. (1980) Calcium uptake in fishes. In: Epithelial transport in the Lower Vertebrates. (Lahlou, B., ed.) Malta: C.U.P., p 121-131.
PAERT, P., KIESSLING, A. & RING, O. (1982) Adrenalin increases vascular resistance in perfused rainbow trout *(Salmo gairdneri* Rich.) gills. Comp.Biochem. Physiol. 72C(1): 107-108.
PAYAN, P., MAYER GOSTAN, N. & PANG, P.K.T. (1981) Site of calcium uptake in the freshwater trout gill. J.Exp.Zool. 216: 345-347.
POTTS, W.T., FOSTER, M.A., RUDY, P.P. & HOWELLS, G.P. (1967) Sodium and water balance in the cichlid teleost, Tilapia mossambica. J.exp.Biol. 47: 461-470.
SHIPLEY, R.A. & CLARK, R.E. (1972) Tracer methods for *in vivo* kinetics. Theory and applications. New York and London: Academic Press.
WENDELAAR BONGA, S.E. & VAN DER MEIJ, J.C.A. (1980) The effect of ambient calcium on prolactin cell activity and plasma electrolytes in *Sarotherodon mossambicus* (Tilapia mossambica). Gen.Comp.Endocrinol. 40: 391-410.
WENDELAAR BONGA, S.E. & MEIS, S. (1981) Effects of external osmolality, calcium and prolactin on growth and differentiation of the epidermal cells of the cichlid teleost *Sarotherodon mossambicus*. Cell Tissue Res. 221: 109-123.
WENDELAAR BONGA, S.E. & FLIK, G. (1982) Prolactin and calcium metabolism in a teleost fish, Sarotherodon mossambicus. In: Comparative Endocrinology of calcium regulation. (Oguro, C. & Pang, P.K.T. eds) Tokyo: Japan Scientific Societies Press, p 21-26.
WENDELAAR BONGA, S.E., LOEWIK, C.J.M. & VAN DER MEIJ, J.C.A. (1983) Effects of external Mg^{2+} and Ca^{2+} on branchial osmotic water permeability and prolactin secretion in the teleost fish *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. 52: 222-231.
WINBERG, G.G. (1956) Rate of metabolism and food requirements of fishes. Fish.Res.Bd.Can.Trans.Ser. 194: 253.

CHAPTER VII

EFFECTS OF LOW AMBIENT CALCIUM LEVELS ON WHOLE-BODY Ca^{2+}-FLUX RATES AND INTERNAL CALCIUM POOLS IN THE FRESHWATER CICHLID TELEOST FISH *OREOCHROMIS MOSSAMBICUS*

ABSTRACT

Calcium fluxes and internal calcium pools were measured in fed, rapidly growing, male tilapia, *Oreochromis mossambicus*, acclimated to ambient Ca^{2+}-concentrations of 0.8 mM (FW) and 0.2 mM (LFW). Plasma calcium levels were slightly and significantly higher in the LFW tilapia, but muscle calcium concentrations were independent of ambient calcium. The LFW fish continued to grow and accumulate calcium although the calcium content of their hard tissues was reduced. The LFW fish had higher Ca^{2+}-influx and Ca^{2+}-efflux than the FW fish. The increase in the influx of Ca^{2+} in LFW fish was, however, substantially greater than the increase in the efflux of Ca^{2+}, giving these fish a more than 4-fold increase in net Ca^{2+}-influx: for a 20-g tilapia net uptake rates of Ca^{2+} from the water were 390 and 1620 $nmol.h^{-1}Ca^{2+}$ for FW and LFW adapted fish, respectively. These values were calculated to represent a substantial portion of the total calcium accumulated by these growing fish. This indicates that even in low-calcium water, tilapia absorb a significant amount of their calcium requirement directly from the ambient medium.

The readily exchangeable bone calcium pool in the FW fish was estimated to be about 7% of the total hard tissue calcium. In the fish acclimated to LFW this percentage increased to about 15% as total hard tissue mineralization decreased. This may indicate that tilapia can increase the mobility of their hard tissue calcium during periods of low calcium stress.

INTRODUCTION

Freshwater fish can and do accumulate calcium directly from the water by absorption across the gills (Simkiss, 1974; Milhaud *et al.*, 1977; Mayer-Gostan *et al.*, 1983) and in at least some species of fish this mode of calcium accumulation is sufficient to maintain normal growth, even when the fish are fed a calcium-deficient diet (Ogino & Takeda, 1976, 1978; Watanabe *et al.*, 1980;

Ichii & Mugiya, 1983). In fact, even when calcium is supplied with the food, direct absorption of calcium from the water via the gills prevails (Berg, 1970). This last uptake mechanisms may be expected to function at full capacity in growing fish which, of course, must be supplied with all other nutritional requirements. Undernourished fish can not be expected to grow and lay down mineralized tissue. Indeed, under conditions of starvation fish show no callus formation in response to bone fracture, as was shown for *Carassius auratus* and *Tilapia macrocephala* (Moss, 1962); carp *(Carassius carassius)* are known to demineralize their scales during starvation (Ichikawa, 1953).

It is well known that calcium-uptake in freshwater fish is considerably higher than that in seawater fish. This is frequently considered to point to the existence in fish of a compensatory calcium uptake mechanism vis-à-vis the availability of calcium in the water. This prompts the question whether the calcium levels in fresh waters - which show considerable variation - determine the magnitude of calcium accumulation in fish which inhabit such environments.

The study reported here was designed to investigate the role of ambient calcium on the net uptake of Ca^{2+} from the water in growing specimens of the freshwater cichlid species *Oreochromis mossambicus*. Fish were acclimated to two levels of calcium in the water. Employing the isotopes ^{47}Ca and ^{45}Ca, Ca^{2+}-influx and Ca^{2+}-efflux were determined separately using a procedure which was developed earlier (Flik *et al.*, 1984). In addition, total as well as readily exchangeable calcium pools of the hard tissues were measured. The rates of uptake of Ca^{2+} from the water were evaluated in relation to growth rates. A possible role for internal stores in the maintenance of calcium homeostasis is discussed.

MATERIALS AND METHODS

Male tilapia, *Oreochromis mossambicus*, weighing 10-30 g were used throughout this study. They were obtained from laboratory stock and were held under conditions as described previously (Flik *et al.*, 1984d).

^{45}Ca and ^{47}Ca were purchased as calcium chloride in aqueous solution (Amersham International plc, England). Specific activities were: for ^{45}Ca, 9.25-37.5 GBq/mol Ca; for ^{47}Ca, >0.74 GBq/mol Ca. Only reagent-grade chemicals (Sigma) were used.

Acclimation

Fish used in the Ca^{2+}-flux studies were acclimated to artificial fresh water pre-

pared from demineralized water and containing (mM): NaCl (3.8), KCl (0.06), $MgSO_4$ (0.2) and $CaCl_2$ (either 0.8 or 0.2); the pH was adjusted with $NaHCO_3$ to 7.4 ± 0.2. The final osmolarity was 8-10 mOsmol/l. Water containing 0.8 mM $CaCl_2$ is referred to as fresh water (FW) and approximates the Nijmegen city tapwater in which the laboratory stock of tilapia is kept and bred. Low-calcium fresh water, containing 0.2 mM $CaCl_2$ is referred to as LFW. Fish were acclimated to LFW by transferring them first from FW to FW containing 0.4 mM $CaCl_2$, and one day later to LFW. Throughout the adaptation period and during the experiments both pH and Ca^{2+}-concentrations were monitored and adjusted as required. Ammonia levels were kept below 2 μM NH_4^+ by charcoal filtration and by changing the water. Fish were maintained in their acclimation medium for at least 10 weeks before the start of flux determinations and were fed their normal ration of food. At the time of flux determinations the fish were growing at a rate of about 3-5% body weight per month and they remained healthy.

Determination of whole-body Ca^{2+}-flux rates

Whole-body Ca^{2+}-flux rates were determined as described in detail earlier (Flik *et al.*, 1984d). A whole-body counter and ^{47}Ca (92.5-370 kBq/l water) as a tracer were used in influx studies. Whole-body efflux rates of Ca^{2+} were determined in two ways. i) Four days after intraperitoneal injection of $^{45}Ca^{2+}$ (37-74 kBq/g fish), on the basis of tracer appearance in small volumes of water (0.5-1 l) and the plasma-^{45}Ca specific activity at the end of the experiment. One-day starved fish, with emptied urinary bladders were used. ii) Four days after $^{47}Ca^{2+}$-injection (92.5-185 kBq/fish) on the basis of apparently linear whole-body tracer loss in tracer-free water and the plasma-^{47}Ca specific activity at that time. In the first type of determination efflux rates of Ca^{2+} essentially represent branchial efflux rates (Flik *et al.*, 1984d). In the second type of determination, efflux rates of Ca^{2+} include urinary and intestinal Ca-secretion. For both groups of fish tracer-uptake, tracer-retention after injection and tracer-loss from the body to the water were plotted to ascertain that the kinetics of these processes permit the application of flux-rate calculations that we developed for FW tilapia (Flik *et al.*, 1984d).

Analytical procedures

Plasma total Ca was determined by atomic absorption spectrophotometry, using 20 mM $LaCl_3$ as a diluent, or with a commercial calcium kit (Sigma) in the case of

^{45}Ca-containing samples.

Three types of bone samples and one muscle sample were taken from every fish. Triplicate samples of 10 scales each were taken from both sides at the mid-lateral region, posterior to the opercular slit. A sample of opercular bone was taken after removing the skin and connective tissue by rubbing with tissue paper. A sample of vertebral bone was taken, whereby adhering soft tissues were removed after pressure-cooking for 1 min as suggested by Fleming (1973). Muscle samples carefully freed of bone and scales were taken from the dorsal region. All tissue samples were dried overnight at 90-100°C, dry weights were determined to the nearest 0.01 mg. The dried samples (5-50 mg) were dissolved in 0.5 ml concentrated HNO_3 at 60°C for 1 h and the sample volume was brought up to 5 ml with doubly-distilled water. Total Ca of tissue digests was determined on 5-50 µl samples with the thymol-blue method of Gindler and King (1972). Calcium references were prepared from a calcium atomic absorption standard solution (1000 µg/ml dilute HCl; Sigma).

For ^{45}Ca analysis, 1 ml of diluted tissue digest of 5-10 l plasma in a volume of 1 ml water, were mixed with 4 ml Aqualuma® (Lumac) and counted in a Rackbeta LSA, equipped with a dpm-program. All samples were assayed in triplicate. Tissue tracer content is presented as relative specific activity (SA_r), which is the ratio of tissues Ca-tracer specific activity (SA_t) to plasma Ca-tracer specific activity (SA_p).

Statistics and notations

Significance of differences between mean values was assessed applying Student's t-test for unpaired observations (α=5%). Significance was accepted at the 2%-level. Linear regression analysis was based on the least-squares method. The symbols, definitions of symbols and units used were taken from Shipley and Clark (1972).

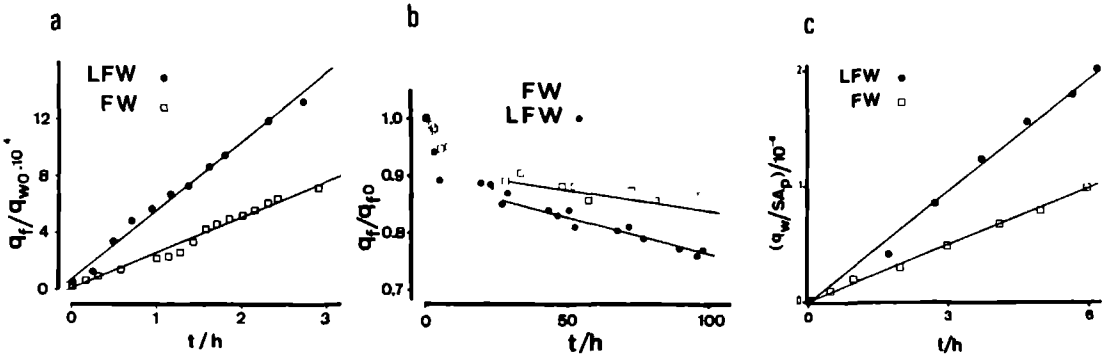
RESULTS

Flux rate determinations

In Fig. 1a, whole-body $^{47}Ca^{2+}$-uptake from the water in FW and LFW tilapia is presented. Under both conditions the increasing whole-body tracer contents showed linearity for a 3-h period. In Fig. 1b, whole-body ^{47}Ca-retention in FW and LFW tilapia is shown. In both cases a rapid decrease in whole-body tracer content

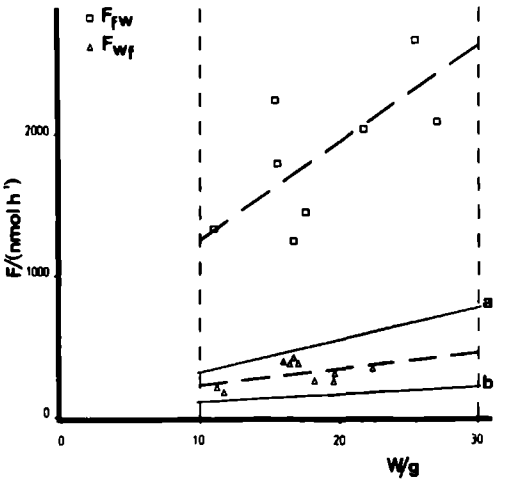
during the first 24 h after injection of the tracer is followed by a steady, slow and apparently linear decrease. LFW-fish however, retained significantly less ^{47}Ca than FW-tilapia. From 24-100 h, the slopes of the tracer retention curves of the FW and LFW tilapia, fitted by linear regression analysis, were significantly different ($P < 0.01$). This clearly shows that LFW tilapia loose tracer faster than FW tilapia. In Fig. 1c, it is shown that tracer appearance in the water was linear over a 6-h period in both cases. The kinetics of the tracer movements in our experimental set-up justify the calculation of Ca^{2+}-flux rates (see: Flik *et al.*, 1984d).

Figure 1. (a) Whole-body $^{47}Ca^{2+}$-uptake from the water expressed as fish tracer content at time t (q_f) relative to the total radioactivity in the water at zero time (q_{w0}). Data concern one FW tilapia and one LFW tilapia only.
(b) Whole-body tracer retention curves for tilapia injected intraperitoneally with $^{47}Ca^{2+}$. Retention is expressed as tracer retained in the body at time t (q_f) divided by tracer in the fish at zero (Injection) time (q_{f0}). Mean values for 7 FW and 5 LFW tilapia are given. Linear regression analysis of the data obtained from 24 - 100 h yielded statistically significant slopes, namely 136 cpm/h for FW tilapia (W = 14.3 ± 0.8 g) and 313 cpm/h for LFW tilapia (W = 18.2 ± 2.1 g).
(c) Tracer appearance in the water upon immersion of $^{45}Ca^{2+}$-injected tilapia in tracer-free water. Water tracer content (q_w) is expressed as the fraction of the plasma ^{45}Ca specific activity of the fish at the end of the experiment (SA_p). Mean values are given for 10 FW tilapia and 6 LFW tilapia.



In Fig. 2, in- and efflux rates of Ca^{2+} in FW and LFW tilapia are compared. Flux rates of Ca^{2+} in FW tilapia determined in the present study did not differ from previously reported values (Flik *et al.*, 1984d). Therefore, Ca^{2+}-influx (F_{fw}) is represented by $F_{fw} = 50W^{0.805}$ nmol/h (line a) and efflux of Ca^{2+} (F_{wf}) by $F_{wf} = 30W^{0.563}$ nmol/h (line b). In the body-weight trajectory studied (W = 10-30 g), Ca^{2+}-influx rates as well as Ca^{2+}-efflux rates in LFW tilapia were significantly higher than the corresponding values for FW tilapia. Fitting the relationships between flux rates and body weights of LFW tilapia by linear regression analysis (dotted lines) and using the forementioned relationships between flux rates and body weight for FW tilapia (that yield essentially straight lines in this particular body weight trajectory), the following formulae were obtained for net fluxes: in FW tilapia $F_{net} = F_{fw} - F_{wf} = 210 + 18\ (W-10)$ nmol/h Ca^{2+} and in LFW tilapia $F_{net} = 1040 + 48\ (W-10)$ nmol/h Ca^{2+}. For a 20-g FW tilapia F_{net} comes to 390 nmol/h Ca^{2+} and for a 20-g LFW tilapia to 1620 nmol/h Ca^{2+}, which indicates a 4.15-fold enhanced uptake of Ca^{2+} from the water in the latter fish.

Figure 2. Comparison of whole-body flux rates in FW and LFW tilapia. In- and efflux rates of Ca^{2+} for FW tilapia are represented by $F_{fw} = 50W^{0.805}$ nmol/h (line a) and by $F_{wf} = 30W^{0.563}$ nmol/h (line b), respectively. For LFW tilapia individual Ca^{2+}-influx rates (□, n=8) and Ca^{2+}-efflux rates (Δ, n=10) are given. In the body weight range from 10 - 30 g the relationships between flux rates and body weight were fitted by linear regression analysis (dotted lines). In this body-weight range, net fluxes of Ca^{2+}, $F_{net} = F_{fw} - F_{wf}$, come to 210 + 18(W-10) nmol/h for FW tilapia and to 1040 + 58(W-10) nmol/h for LFW tilapia.



Extrabranchial Ca^{2+}-efflux rates

As shown in Fig. 1b, tracer loss from the body, although an exponential process, can be satisfactorily analyzed by linear regression analysis of the data obtained between 24 and 100 h. On the basis of tracer losses during this period and plasma ^{47}Ca specific activity at 62 h (halfway through this period) total Ca^{2+}-efflux rates were calculated. In a previous paper it was shown that plasma tracer contents over this period decreased linearly and concurrently with total body tracer contents (Flik *et al.*, 1984d). For FW tilapia the measured total efflux rate of Ca^{2+} was 208 ± 71 nmol/h (n=5; W=14.3 ± 0.8 g). The calculated branchial efflux rate of Ca^{2+}, calculated according to $F_{wf} = 30W^{0.563}$ nmol/h, was 179 ± 10 nmol/h. For LFW tilapia measured total efflux rate of Ca^{2+} was 557 ± 45 nmol/h (W=18.2 2.1 g). This last value is significantly higher ($P < 0.02$) than the measured branchial Ca^{2+}-efflux rate which came to 383 ± 67 nmol/h (W=18.5 ± 3.2 g). Extrabranchial efflux rates of Ca^{2+} calculated as the difference in mean total and branchial efflux rates, come to 29 nmol/h for FW tilapia and to 178 nmol/h for LFW tilapia, or 14% and 31% of the total Ca^{2+}-efflux, respectively. For these calculations it was assumed that the specific activity of the ^{47}Ca lost from the body equalled the plasma-^{47}Ca specific activity.

Tissue-Ca analyses

As shown in Table I, LFW-tilapia showed a slightly, but significantly higher plasma total Ca level than FW tilapia.
In LFW tilapia the bone Ca-content was significantly lower than in FW tilapia, in all three types of bones. This difference was more pronounced in the skeletal bone (vertebrae; 13%) and scalar bone (11.4%) than in the dermal bone (operculum; 6.7%). Ca-content of muscle on dry-weight basis did not differ significantly between the two groups of fish.

Tissue tracer contents were determined at the completion of the efflux experiments, i.e. 80 ± 3 h after $^{45}Ca^{2+}$-injection. Relative specific activities (SA_r) for vertebrae, opercula and scales were significantly higher in LFW tilapia than in FW tilapia (Table I). This difference in SA_r-value was most pronounced in scales (142%) and amounted to 107% and 108% in vertebrae and opercula, respectively. SA_r-values for muscle did not differ between the two groups and were not significantly different from plasma SA-values.

Table I. Ca content and relative ^{45}Ca content (SA_r) of several tissues of FW and LFW tilapia, determined 80 ± 3 h after tracer injection.

Ca-content of muscle and bony tissues are compared on dry-weight basis. Values for the relative tracer content of the tissues are expressed as tissues ^{45}Ca specific activity (SA_t) relative to plasma ^{45}Ca specific activity (SA_p). Mean values ± S.E. are given for 7 FW and 12 LFW tilapia.

			SA_r ($100 \times SA_t/SA_p$)	
Fish:	FW	LFW	FW	LFW
Tissue:				
Plasma	2.77 ± 0.22	2.99 ± 0.13§	100	100
Muscle	$(12 \pm 6).10^{-3}$	$(13 \pm 5).10^{-3}$	94.7 ± 25.5	94.8 ± 33.3
Bone:				
Vertebrae	5.40 ± 0.41	4.70 ± 0.48(††)	6.7 ± 2.0	13.9 ± 5.6(††)
Operculum	6.83 ± 0.31	6.37 ± 0.36(†††)	5.8 ± 1.2	12.1 ± 4.4(††)
Scales	5.36 ± 0.25	4.75 ± 0.43(††)	8.6 ± 1.7	20.8 ± 5.6(†)

†) $P < 0.001$ ††) $P < 0.01$ †††) $P < 0.02$ § Expressed in mM

Table II presents the results obtained for 4 fish adapted to either FW or LFW and whose total skeletal, dermal and scalar bone were collected to determine the relative sizes of these "subpools" of bone. The sizes of these subpools presented as a percentage of the total bone-Ca pool (Q_{bone}) were not significantly different between the two samples of fish. Neither was a difference observed with respect to Q_{bone}, expressed per body wet-weight.

Table II. Comparison of Ca-containing compartments in tilapia acclimated for 12 weeks to FW and LFW conditions.

The total amount of the skeletal bone compartment (Q_{skel}), of the dermal bone compartment (Q_{derm}), of the scalar bone compartment (Q_{scal}), of the total bone compartment ($Q_{bone} = Q_{skel} + Q_{derm} + Q_{scal}$), of the soft tissue compartment (Q_{soft}) and of the complete fish ($Q_f = Q_{bone} + Q_{soft}$) are expressed in mmol Ca. Bone Ca-content on dry-weight basis (Q_{bone}/W_{bone}) is expressed in mmol/g.

Total bone mass is presented as the total bone dry weight relative to the body weight of the fish ($W_{bone}/(10^{-2}W_f)$). In all cases mean values ± S.E. concern 4 fish.

		FW tilapia (W = 12.1 ± 1.2 g)	LFW tilapia (W = 15.3 ± 1.8 g)
Compartment size	: Q_{skel}	1.56 ± 0.14 (41.3*)	1.87 ± 0.03 (40.3*)
	Q_{derm}	1.31 ± 0.25 (34.8*)	1.62 ± 0.12 (35.1*)
	Q_{scal}	0.90 ± 0.13 (23.9*)	1.14 ± 0.11 (24.6*)
	Q_{soft}	0.24 ± 0.04 (5.9§)	0.27 ± 0.05 (5.6§)
	Q_{bone}	3.76 ± 0.49 (94.1§)	4.63 ± 0.14 (94.4§)
	Q_f	4.00	4.90
Ca-content	: Q_{bone}/W_{bone}	5.75 ± 0.02	5.22 ± 0.05†
Relative bone mass:	$W_{bone}/(10^{-2}.W_f)$	5.38 ± 0.15	5.85 ± 0.25††

*: % of Q_{bone}; §: % of Q_{fish}; †: $P < 0.001$; ††: $P < 0.02$

Total bone mass however, expressed as bone dry-weight per body wet-weight, was significantly higher in LFW than in FW tilapia ($P < 0.02$). The total bone Ca-content differed significantly between FW and LFW tilapia, averaging 5.75 ± 0.02 mmol/g for FW fish and 5.22 ± 0.05 mmol/g for LFW fish ($P < 0.001$). For FW tilapia the difference between total body Ca (calculated as $Q_f = 357.5W^{0.965} = 3.98 \pm 0.38$ mmol Ca) and Q_{bone} (= 3.76 ± 0.49 mmol Ca) yields the soft-tissue Ca-pool (Q_{soft} = 0.22 mmol Ca), the latter being 5.42% of Q_f. This value for Q_{soft} did not differ significantly from measured values for the soft tissue compartment of 0.24 ± 0.04 mmol Ca, which is 5.9% of Q_f. For LFW tilapia Q_f can be calculated then on the basis of tissue Ca-contents and the relative sizes of the respective subpools presented in Table II.

Calcium accumulation in tilapia acclimated to FW or LFW

Table III presents data on growth and Ca accumulation in tilapia acclimated to FW or LFW. Three groups are considered: one FW-group and two different groups of fish that were acclimated to LFW conditions. In all three cases significant weight increase and accumulation of Ca in the body occurred during the acclimation period, as indicated by the increase in mean body weight and mean total fish calcium pools. Mean body accumulation rates of Ca calculated as $\Delta Q_f/\Delta t$ are 383

nmol/h Ca for FW fish and 616 and 510 nmol/h Ca for the two groups of LFW fish.

Table III. Growth and Ca-accumulation in FW and LFW tilapia.

W_0 represents the body weight at the start of the acclimation period; mean values ± S.E. are given. Δt refers to the duration of the acclimation period. ΔW represents the mean increase in body weight per fish over the pertinent period. ΔQ_f refers to the calculated increase in total body Ca per fish over the pertinent period.

Fish	n	W_0 (g)	Δt (h)	ΔW (g)	ΔQ_f (mmol Ca)
FW	7	19.95 ± 2.03	1464	1.82	0.561
LFW	7	19.40 ± 6.42	1944	3.66	1.197
LFW	5	24.10 ± 3.75	2424	3.60	1.237

DISCUSSION

Six major conclusions can be drawn from the data presented in this study.

1. Tilapia well-adapted to a low-calcium medium showed a net uptake of Ca from the water as indicated by growth and accumulation of Ca in the body.
2. Net branchial Ca^{2+}-influx rates in these fish increased more than four-fold.
3. Both the branchial and extrabranchial Ca^{2+}-efflux rates underwent significant increases during the acclimation process.
4. The low-calcium adapted tilapia showed slight but significant hypercalcemia when compared with tilapia in normal fresh water.
5. The acclimation to low-calcium conditions was accompanied by a decrease in bone Ca-content.
6. Finally, the pool of readily exchangeable Ca in bone tissue of tilapia well-adapted to low-calcium was significantly bigger than that in tilapia from normal fresh water.

Ca^{2+}-flux rates

Tilapia acclimated to low-calcium conditions accumulate Ca in their body, which indicates that this species is capable of establishing a positive Ca balance even

after a four-fold reduction of the calcium in their environment. It has been shown, however, that in tilapia bone demineralization and loss of total body calcium take place in the first two weeks after transferring them to a low-calcium environment (Wendelaar Bonga *et al.*, 1984). This suggests that the positive Ca balance, which was established after ten weeks, was re-established after a period of negative Ca balance. Apparently, with regard to Ca metabolism the acclimation to LFW conditions is a relatively slow process. Indeed, in three fish exposed for only one week to LFW, Ca^{2+}-influx rates were still roughly the same as those of FW tilapia (unpublished result). The question arising then is: how do the Ca^{2+}-flux rates observed in tilapia compare to values reported in the literature for other species by linear extrapolation to fluxes per h per kg fish, it turns out that the Ca^{2+}-flux rates in tilapia are high. The following influx rates (µmol/h.kg fish) were calculated. For tilapia adapted to FW and LFW: 28 and 116, respectively; for rainbow trout, *Salmo gairdneri*, and bullheads, *Ictalurus nebulosus*, adapted to ambient Ca-levels of 0.085 - 0.325 mM: 5 - 7.5 (Höbe *et al.*, 1984); for the goldfish, *Carassius auratus:* 15 (Berg, 1968, 1970); for the killifish, *Fundulus heteroclitus:* 32.5 (Pang *et al.*, 1980) and 10 - 50 (Mayer-Gostan *et al.*, 1983); and for *Fundulus kansae:* 27 (Fleming, 1973). Efflux rates presented in this way come to 8.1 and 15.7 for FW and LFW tilapia, respectively; to 5 - 7.5 for rainbow trout and to 1 - 5 for bullheads (Höbe *et al.*, 1984). Influx rates of Ca^{2+} in FW tilapia are of the same order only as the values for the two *Fundulus*-species. The values for LFW tilapia exceed every value reported for directly measured flux rates in fish. However, when comparing Ca^{2+}-flux rates in tilapia to Ca^{2+}-flux rates reported for other species a comment must be made concerning the presentation of Ca^{2+}-fluxes rates in the literature. To our knowledge it has not been recognized before that Ca^{2+}-flux rates in fish are not necessarily directly related to body weight. Hence, flux rates linearly extrapolated to fluxes per e.g. 0.1 or 1 kg fish may be misleading, especially when small fish are used. For example, when the proper relationships are observed (Flik *et al.*, 1984d) net Ca^{2+}-influx in a 20-g tilapia in FW is calculated to $F_{net} = F_{fw} = 558\text{-}162\text{-}396$ nmol/h, or = 13000-1466 = 11534 nmol/h for a - hypothetical - tilapia of 1 kg. Values obtained by linear extrapolation (multiplying by 50) would yield F_{net} = 27900-8100 = 19800 nmol/h per kg tilapia and overestimate true flux rates.

The very high value for net influx rates in LFW tilapia that we obtained by direct flux measurement compares well with an uptake rate of Ca^{2+} from the water derived from growth rates reported by Ichii and Mugiya (1983) for rapidly growing goldfish, fed a calcium deficient diet. The most important conclusion from our results is that in tilapia the branchial calcium uptake system has a great capa-

city for adaptation and can supply almost all of the fish' calcium requirements and even can permit growth in relatively soft water. It is also evident from our results that to assess uptake of Ca^{2+} from the water by flux rate determination, both influx and efflux rates should be determined, as the magnitude and routes of Ca^{2+}-movement depend on ambient Ca^{2+}-concentrations: branchial and extrabranchial efflux rates as a percentage of total body efflux rates were estimated to represent 86% and 14% in FW tilapia and 69% and 31% in LFW tilapia.

As we have discussed before, integumental Ca^{2+}-exchange may involve both transcellular and paracellular routes (Flik *et al.*, 1984d). For transcellular Ca^{2+}-uptake in fish gills we recently proposed a model on the basis of our studies on Ca^{2+}-transporting ATPase in plasma membranes of branchial epithelium (Flik *et al.*, 1983, 1984a). According to this model, Ca^{2+} to be transported from the water to the blood enters the cell passively down an electrochemical gradient, is buffered in the cytosol by Ca^{2+}-binding proteins and subsequently pumped to the blood by an active Ca^{2+}-transport mechanism. We suggest now that at least three events are involved in the changes in integumental Ca^{2+}-fluxes, when tilapia are acclimated to LFW.

First, it has been demonstrated in tilapia that an inverse relationship exists between ambient Ca^{2+} and prolactin cell activity (Wendelaar Bonga *et al.*, 1983, 1984a), which implies that prolactin secretion is enhanced in the LFW tilapia. Prolactin stimulates Ca^{2+}-tracer influx in American eel gill arches (Ma & Copp, (1981), stimulates transport Ca^{2+}-ATPase activity in American eel gill plasma membranes (Flik *et al.*, 1984d) and stimulates uptake of Ca^{2+} from the water in intact tilapia (Flik *et al.*, 1984e). Therefore, the enhanced prolactin secretion that occurs under LFW conditions may stimulate Ca^{2+}-transport mechanisms in the branchial epithelium and by so doing increase the Ca^{2+}-transport capacity of the gills. Such an adaptation, in concert with increased entry of Ca^{2+} at the apical membranes probably accounts for the observed increased transcellular Ca^{2+}-influx in the gills.

Secondly, in LFW tilapia the chloride cell density is tripled, when compared to FW tilapia (unpublished results). We consider the chloride cells of the gills as their Ca^{2+}-transporting units (Flik *et al.*, 1984a). Thus, an increase in chloride cell density in the branchial epithelium leads to an increase of transcellular influx capacity.

Thirdly, a decrease in ambient Ca^{2+} causes enhanced permeability to monovalent ions (Dharmamba & Maetz, 1975) and osmotic water permeability (Wendelaar Bonga & Van der Meij, 1981; Wendelaar Bonga *et al.*, 1983) of tilapia gills as well as of the gills of Japanese eel, *Anguilla japonica* (Ogawa, 1974; Ogasaware & Hirano, 1984a), *Fundulus kansae* (Potts & Fleming, 1970), rainbow trout, *Salmo gairdneri* (Ogawa, 1974) and brown trout, *Salmo trutta* (Oduleye, 1975). Although no data are

available in the literature on gill Ca^{2+}-permeability, It is relevant to mention that in chick gut the permeability to Ca^{2+} of apical membranes is determined by and negatively correlated with mucosal Ca^{2+}-concentrations (Ebel & Guenther, 1980; Bikle *et al.*, 1984). If in tilapia similarly a decrease in ambient Ca^{2+} would enhance permeability to Ca^{2+} of the apical plasma membranes of the branchial epithelium, this process would facilitate Ca^{2+}-permeation at the apical membranes and thus promote transcellular Ca^{2+}-influx rates in the gills. However, increase permeability may also lead to increased Ca-loss, Such loss has been described e.g. for paracellular secretion of Ca^{2+} in rat ileal epithelium (Nellans & Kimberg, 1978, 1979), that is determined by and negatively correlated with luminal Ca^{2+}-concentrations. By analogy, low calcium concentrations in the ambient water of tilapia would allow intercellular Ca^{2+} to diffuse out of the animal. Thus, branchial efflux rates of Ca^{2+}, following paracellular routes could be increased as a result of lowered ambient Ca^{2+}. The above mentioned increase in chloride cells in LFW fish may, however, also contribute to an increase in Ca^{2+}-efflux, since it implies extension of the paracellular flux route. Recently, Ogasawara and Hirano (1984b) reported for *Anguilla japonica* that gill permeability to water is positively correlated with the number of chloride cells in the gills and that the number of junctional complexes in the epithelium may determine its permeability to water.

Our results on increased uptake rates of Ca^{2+} from the water in LFW tilapia contrast with the results of Berg (1968, 1970) on the goldfish adapted to low-calcium water. He concluded that branchial exchange rates of Ca^{2+} in this species are independent of ambient Ca^{2+}-levels. Under low-calcium conditions intestinal absorption of Ca became more important relative to branchial Ca^{2+}-uptake. Apparently, the goldfish adjusts its intestinal calcium absorption in stead of its branchial Ca^{2+}-uptake, in a soft water environment. For two other species of freshwater fish, viz. bullheads and rainbow trout, it was reported that whole-body calcium exchange rates were largely independent of ambient Ca^{2+} (Höbe *et al.*, 1984). However, altered influx rates of Ca^{2+} as an adaptive response to varying ambient Ca^{2+}-levels have been reported earlier for *Fundulus heteroclitus* (Mayer-Gostan *et al.*, 1983). Thus such adaptive responses of the branchial calcium uptake system seem of wider occurrence.

The observation of increased extrabranchial Ca^{2+}-efflux rates in LFW tilapia reminds of our observations on increased integumental osmotic water permeability at low ambient Ca^{2+} (Wendelaar Bonga & Van der Meij, 1981). Increased osmotic water uptake or low ambient Ca^{2+} may enhance urine production. Increased urine production leads to extra Ca^{2+}-loss from the body in American eels (Fenwick, 1981).

Hence, increased urine production as a response to enhanced osmotic water uptake in LFW tilapia could explain, at least partly, the increase in extrabranchial Ca^{2+}-efflux under these conditions.

Plasma Ca levels

Our finding of an elevated plasma Ca content in tilapia ten weeks after the start of the fishes' acclimation to low-calcium water confirms a report by Wendelaar Bonga *et al.* (1984). In view of the fact that tilapia five days after transferring the fish to a low-calcium environment show a significant hypocalcemia (Wendelaar Bonga *et al.*, 1984), it appears that in this species "maintenance" of plasma Ca in spite of reduced ambient Ca^{2+} is preceeded by an initial drop in plasma Ca. Most likely the above mentioned restauration of plasma Ca-levels is mediated by an enhanced production of the hormone prolactin. Two arguments in favor of this presumption may be mentioned. First, low ambient Ca^{2+} stimulates endogenous prolactin secretion in tilapia (Wendelaar Bonga *et al.*, 1984). Secondly, administration of prolactin induces hypercalcemia in several species of freshwater teleosts: the stickleback, *Gasterosteus aculeatus*, tilapia and American eels (Wendelaar Bonga & Flik, 1982; Flik *et al.*, 1984b).

Internal Ca-stores

Reducing ambient Ca^{2+}-concentrations changed the amount of calcium in the soft tissue compartment from 5.9% to 5.6% of the total amount of Ca in the fish, a change which is not statistically significant. Comparable values for the soft tissue Ca compartment size of other fish under freshwater conditions are: 6% for the goldfish (Berg, 1968) and 3.2% for *Fundulus kansae* (Fleming *et al.*, 1973). Muscle Ca-content in tilapia seems not affected by changes in plasma Ca-levels. The values for bone and muscle Ca-contents of tilapia adapted to FW are approximately the same as those reported earlier (Wendelaar Bona & Flik, 1982; Wendelaar Bonga & Lammers, 1982). Tilapia kept in LFW showed decreased Ca contents of the major internal Ca stores. The fish used in the present study increased their total body calcium pool under LFW conditions. However, bone calcium contents in these fish were lower than the levels observed in FW tilapia. Thus, although tilapia increase the uptake of Ca^{2+} from the water and re-establish a positive calcium balance under LFW conditions, the degree of bone mineralization is maintained at a lower level. The fact that in LFW tilapia also phosphate-metabolism may be affected by ambient Ca^{2+}-levels could give at least a partial explanation

for this phenomenon. Mobilization of Ca from acellular bone has been reported for *Fundulus kansae* (Brehe & Fleming, 1976), goldfish and killifish (Mugiya & Watabe, 1977), *Lepomis macrochirus* (Weis & Watabe, 1978), *Tilapia macrocephala* (Weis & Watabe, 1979) and for tilapia (Urasa *et al.*, 1984). Both Weis and Watabe, and Urasa and coworkers came to the conclusion that the need for phosphate and not Ca was the primary trigger for bone demineralization. Rodgers (1984) reports for brook trout, *Salvelinus fontinalis*, that low ambient Ca^{2+}-levels impair absorption of dietary Ca^{2+} and that mobilization of bone minerals from scales and fins occurs under such conditions. An attractive possible explanation for the demineralization of bone at low ambient Ca^{2+} would be that calcium and phosphate resorption in the gut are impaired, which urges the fish to mobilize bone mineral (or limit bone mineralization, or both) to provide for its phosphate requirements. The capacity to specifically mobilize calcium-phosphate but not calcium-carbonate reported for *Lepomis macrochirus* after estrogen treatment (Weis & Watabe, 1978) supports this hypothesis.

The degree of demineralization was higher in vertebral and scalar bone than in opercular bone. This can be related to two differences in histophysiology of these bones. First, vertebrae and scales are a cancellous, less dense type of bone than the opercular bone (Moss, 1963; Lanzing, 1976). In those cases where shifts in physico-chemical Ca^{2+}-exchange processes at the bone surface constitute the basis of bone resorption, Ca^{2+}-mobilization occurs more intensily in the less dense type of bone (Amprino, 1952a, 1952b). Secondly, Rowland (1966) has shown that bone structures with the greatest exposure to circulating fluids are the primary sites for Ca^{2+}-exchange processes between blood and bone. Vogel (1982) has shown that the scales of tilapia are very well provided with the so-called secondary vessel system. This system that branches off from the primary blood vessels in the skin covering the scales, could allow for an efficient exchange of minerals at the surface of the bone.

The SA_r-values determined for the various tissues give an indication of the readily exchangeable Ca-pool of these tissues. Four days after injection of the tracer, SA_r-values for muscle approximated the value 100, which means that this tissue exchanged its Ca rather rapidly and completely with the plasma. Brehe and Fleming (1976) came to the same conclusion for the Ca-exchange rate of the soft tissue compartment of *Fundulus kansae*. SA_r-values for bones in FW tilapia ranged from 5.83% (operculum) to 8.61% (scales). These values further parallel the values for bone Ca-content, which corroborates the thesis that the bone density, at least partly, determines the size of the exchangeable pool. Assuming that four days after tracer-injection bone SA_r-values represent the percentage readily ex-

changeable Ca of the bone, we can calculate that the readily exchangeable Ca for FW and LFW tilapia is 12% and 19%, respectively. In FW tilapia the soft tissue compartment and total bone compartment provide for 47% and 53%, respectively, of the readily exchangeable Ca, and in LFW tilapia these figures come to 27% and 73%, respectively. Apparently, in LFW fish the bone provides for an important enlarged readily-exchangeable Ca-pool. This increase in readily-exchangeable Ca in the body of LFW tilapia may fulfil a calcium-buffer function under conditions of increased whole body turnover.

REFERENCES

AMPRINO, R. (1952a) Autoradiographic analysis of the distribution of labelled Ca and P in bones. Exper. 8: 20.

AMPRINO, R. (1952b) Further experiments on the fixation *in vitro* of radiocalcium to sections of bone. Exper. 8: 380.

BERG, A. (1968) Studies on the metabolism of calcium and strontium in freshwater fish. I. Relative contribution of direct and intestinal absorption. Me.Ist.Ital. Idrobiol. 23: 161-196.

BERG, A. (1970) Studies on the metabolism of calcium and strontium in freshwater fish. II. Relative contribution of direct and intestintal absorption in growth conditions. Me.Ist.Ital.Idrobiol. 26: 241-255.

BIKLE, D.D., ZOLOCK, D.T. & MORRISSEY, R.L. (1981) Action of vitamin D on intestinal calcium transport. Ann. NY Acad.Sci. 372: 481-501.

BREHE, J.E. & FLEMING, W.R. (1976) Calcium mobilization from acellular bone and effects of hypophysectomy on calcium metabolosm in *Fundulus kansae*. J.Comp. Physiol. 110: 159-169.

EBEL, H. & GUENTHER, T. (1980) Magnesium metabolism: a review. J.Clin.Chem.Clin. Biochem. 18: 257-270.

FENWICK, J.C. (1981) The renal handling of calcium and renal Ca^{2+}, Mg^{2+}-activated adenosine triphosphatase activity in freshwater- and seawater-acclimated North American eels (*Anguilla rostrata* LeSueur). Can.J.Zool. 59: 478-484.

FLEMING, W.R. (1973) Electrolyte metabolism of teleosts, including calcified tissues. In: Chemical Zoology, Vol. VIII (Florkin, M. and Scheer, B.T., eds) New York, Academic Press: 471-508.

FLEMING, W.R., BREHE, J. & HANSON, R. (1973) Some complicating factors in the study of the calcium metabolism of teleosts. Amer.Zool. 13: 793-797.

FLIK, G., WENDELAAR BONGA, S.E. & FENWICK, J.C. (1983) Ca^{2+}-dependent phosphatase and ATPase activities in eel gill plasma membranes -I. Identification of Ca^{2+}-activated ATPase activities with non-specific phosphatase activities. Comp. Biochem.Physiol. 76B: 745-754.

FLIK, G., WENDELAAR BONGA, S.E. & FENWICK, J.C. (1984a) Ca^{2+}-dependent phosphatase and Ca^{2+}-dependent ATPase activities in plasma membranes of eel gill epithelium -II. Evidence for transport high-affinity Ca^{2+}-ATPase.

FLIK, G., WENDELAAR BONGA, S.E. & FENWICK, J.C. (1984b) Ca^{2+}-dependent phosphatase and Ca^{2+}-dependent ATPase activities in plasma membranes of eel gill epithelium -III. Stimulation of branchial high-affinity Ca^{2+}-ATPase during prolactin induced hypercalcemia. Comp.Biochem.Physiol. In Press.

FLIK, G., FENWICK, J.C., KOLAR, Z., MAYER-GOSTAN, N. & WENDELAAR BONGA, S.E. (1984d) Whole body Ca^{2+}-flux rates in the cichlid teleost fish *Oreochromis mossambicus*, adapted to fresh water. Amer.J.Physiol. (submitted).

GINDLER, E.M. & KING, J.D. (1972) Rapid colorimetric determination of calcium in biological fluids with methylthymol Blue. Am.J.Clin.Pathol. 58: 376-382.
HÖBE, H., LAURENT, P. & McMAHON, B.R. (1984) Whole body calcium flux rates in freshwater teleosts as a function of ambient calcium and pH levels: a comparison between the euryhaline trout, *Salmo gairdneri* and stenohaline bullhead, *Ictalurus nebulosus*. J.exp.Biol.: In Press.
ICHII, T. & MUGIYA, Y. (1983) Effects of a dietary deficiency in calcium on growth and calcium uptake from the aquatic environment in the goldfish, *Carassius auratus*. Comp.Biochem.Physiol. 74A: 259-262.
ICHIKAWA, R. (1953) Absorption of fish scale caused by starvation. Rec.oceanogr. Wks Japan 1: 101-104.
LANZING, W.J.R. & WRIGHT, R.G. (1976) The ultrastructure and calcification of the scales of *Tilapia mossambica* (Peters). Cell Tiss.Res. 167: 37-47.
MA, S.W.Y. & COPP, D.H. (1981) Prolactin and calcium metabolism in teleosts. In: Cohn, D.V., Talmage, R.V. and Matthews, J.L. (eds) Hormonal control of calcium metabolism. Excerpta medica, Amsterdam, 423.
MAYER-GOSTAN, N., BORNANCIN, M., DeRENZIS, G., NAON, R., YEE, J.A., SHEW, R.L. & PANG, P.K.T. (1983) Extraintestinal calcium uptake in the killifish, *Fundulus heteroclitus*. J.exp.Zool. 227: 329-338.
MILHAUD, G., RANKIN, J.C., BOLIS, L. & BENSON, A.A. (1977) Calcitonin: Its hormonal action on the gill. Proc.Natl.Acad.Sci. 74(10): 4693-4696.
MOSS, M.L. (1962) Studies of the acellular bone of teleost fish. II. Response to fracture under normal and acalcemic conditions. Acta anat. 48: 46-60.
MOSS, M.L. (1963) The biology of acellular teleost bone. Ann. NY Acad.Sci. 109: 227-350.
MUGIYA, Y. & WATABE, N. (1977) Studies on fish scale formation and resorption -II effect of estradiol on calcium homeostasis and skeletal tissue resorption in the goldfish, *Carassius auratus* and the killifish, *Fundulus heteroclitus*. Comp.Biochem.Physiol. 57A: 197-202.
NELLANS, H.N. & KIMBERG, D.V. (1978) Cellular and paracellular calcium transport in rat ileum: effects of dietary calcium. Amer.J.Physiol. 235: E726-E737.
NELLANS, H.N. & KIMBERG, D.V. (1979) Anomalous calcium secretion in rat ileum: role of paracellular pathway. Amer.J.Physiol.: E473-E481.
ODULEYE, S.O. (1975) The effects of calcium on water balance of the brown trout *Salmo trutta*. J.exp.Biol. 63: 343-356.
OGASAWARA, T. & HIRANO, T. (1984a) Effects of prolactin and environmental calcium on osmotic water permeability of the gills in the eel, *Anguilla japonica*. Gen.Comp.Endocrinol. 53: 315-325.
OGASAWARA, T. & HIRANO, T. (1984b) Changes in osmotic water permeability of the eel gills during seawater and freshwater adaptation. J.Comp.Physiol. 154: 3-11.
OGAWA, M. (1974) The effects of bovine prolactin, sea water and environmental calcium on water influx in isolated gills of the euryhaline teleosts, *Anguilla japonica* and *Salmo gairdneri*. Comp.Biochem.Physiol. 49A: 545-553.
OGINO, C. & TAKEDA, H. (1976) Mineral requirements in fish-III. Calcium and phosphorus requirements in carp. Bull.Jap.Soc.Sci.Fish. 42: 793-799.
OGINO, C. & TAKEDA, H. (1978) Requirements of rainbow trout for dietary calcium and phosphorus. Bull.Jap.Soc.Sci.Fish. 44: 1019-1022.
PANG, P.K.T., GRIFFITH, R.W., MAETZ, J. & PIC, P. (1980) Calcium uptake in fishes. In: Epithelial transport in the lower vertebrates (ed. B. Lahlou) Cambridge University Press: 121-132.
PAYAN, P., MAYER-GOSTAN, N. & PANG, P.K.T. (1981) Site of calcium uptake in the freshwater trout gill. J.exp.Zool. 216: 345-347.
POTTS, W.T.W. & FLEMING, W.R. (1970) The effects of prolactin and divalent ions on the permeability to water of *Fundulus kansae*. J.exp.Biol. 53: 317-327.
RODGERS, D.W. (1984) Effects of ambient pH and calcium concentration on growth and calcium dynamics of brook trout, *Salvelinus fontinalis*. Can.J.Fish Aqua Sci. 113. Submitted.

ROWLAND, R.E. (1966) Exchangeable bone calcium. Clin.Orthop.Rel.Res. 49: 233-248.
SIMKISS, K. (1974) Calcium metabolism of fish in relation to ageing. In: Ageing of fish (ed. Begenal, T.B.), Unwin Brothers, Old Woking: 1-12.
SHIPLEY, R.A. & CLARK, R.E. (1972) Tracer methods for *in vivo* kinetics- Theory and applications. New York and London, Academic Press.
URASA, F., FLIK, G. & WENDELAAR BONGA, S.E. (1984) Mobilization of bone minerals by estrogens during ovarian growth in the tilapia *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. 53(3): 495.
VOGEL, 1982 personal communication.
WATANABE, T., MURAKAMI, A., TAKEUCHI, L., NOSE, T. & OGINO, C. (1980) Requirement of chum salmon held in fresh water for dietary phosphorus. Bull.Jap.Soc.Sci. Fish. 46: 361-367.
WEISS, R.E. & WATABE, N. (1978a) Studies on the biology of fish bone -II. Bone resorption after scale removal. Comp.Biochem.Physiol. 60A: 207-211.
WEISS, R.E. & WATABE, N. (1979) Studies on the biology of fish bone III. Ultrastructure of osteogenesis and resorption in osteocytic (cellular) and anosteocytic (acellular) bones. Calcified Tissue Int. 28: 43-56.
WENDELAAR BONGA S.E. & VAN DER MEIJ, J.C.A. (1981) The effect of ambient osmolarity and calcium on prolactin cell activity and osmotic water permeability of the gills in the teleost *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. 43: 432-442.
WENDELAAR BONGA, S.E. & LAMMERS, P.I. (1982) Effects of calcitonin on ultrastructure and mineral content of bone and scales of the cichlid teleost *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. 48: 60-70.
WENDELAAR BONGA, S.E. & FLIK, G. (1982) Prolactin and calcium metabolism in a teleost fish. In: Comparative Endocrinology of Calcium Regulation (Oguro, C. and Pang, P.K.T., eds) Tokyo, Japan Scientific Societies Press: 21-26.
WENDELAAR BONGA, S.E., FLIK, G. & FENWICK, J.C. (1984) Prolactin and calcium metabolism in fish: effects on plasma calcium and high-affinity Ca^{2+}-ATPase in gills. In: Endocrine control of bone and calcium metabolism (D.V. Cohn, J.T. Potts Jr., T. Fujita, eds) Elsevier Science Publishers B.V.: 188-190.
WENDELAAR BONGA, S.E., LOEWIK, C.W.G.M. & VAN DER MEIJ, J.C.A. (1983) Effects of external Mg^{2+} and Ca^{2+} on branchial osmotic water permeability and prolactin secretion in the teleost *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. 52: 222-231.
WENDELAAR BONGA, S.E., FLIK, G., LOEWIK, C.W.G.M. & VAN EYS, G.J.J.M. (1984a) Environmental control of prolactin secretion in the teleost fish *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. In Press.
WENDELAAR BONGA, S.E., VAN DER MEIJ, J.C.A. & FLIK, G. (1984b) Prolactin and acid stress in the teleost *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. In Press.

CHAPTER VIII

SOME EFFECTS OF OVINE PROLACTIN ON Ca^{2+}-UPTAKE AND DISTRIBUTION OF CALCIUM IN THE FRESHWATER CICHLID TELEOST FISH, *OREOCHROMIS MOSSAMBICUS*

ABSTRACT

Ovine prolactin stimulated the rate of uptake of calcium from the water, produced frank hypercalcemia, and increased total bone calcium content in fed, rapidly growing freshwater male tilapia, *Oreochromis mossambicus*. It did not, however, alter the size of the readily exchangeable bone calcium pool. The increase in calcium accumulation resulted primarily from an increase in branchial calcium influx, but also from a decrease in calcium efflux. It is concluded that prolactin exerts an important control over calcium exchange between freshwater teleosts and their environment and that by so doing this hormone facilitates indirectly bone mineralization.

INTRODUCTION

Prolactin functions as a hypercalcemic hormone in teleost fish. Injection of mammalian prolactin induces frank hypercalcemia in tilapia, sticklebacks, rainbow trout, goldfish and American and European eel (Wendelaar Bonga & Flik, 1982; Wendelaar Bonga *et al.*, 1984a). Conversely, hypophysectomized killifish become hypocalcemic and exhibit tetanic seizures in Ca^{2+}-deficient sea water; these disturbances of the calcium metabolism are overcome either by supplying calcium to the water or treating the fish with exogenous prolactin (Pang *et al.*, 1973, 1978). The effectiveness of endogenous prolactin is evident from the observation that ectopic transplants of homologous prolactin lobes into tilapia exert a hypercalcemic influence (Wendelaar Bonga & Flik, 1982). Moreover, in this last species an inverse relationship exists between levels of ambient calcium and prolactin cell activity (Wendelaar Bonga *et al.*, 1983b). While these observations clearly suggest that prolactin has a hypercalcemic effect in teleost fish, the mechanisms involved in this hormone's action are still poorly understood.

It is well-established that fish have a remarkable capacity to extract calcium directly from their surrounding aqueous environment (Simkiss, 1974). Indeed, it

has been estimated that at least 80% of the calcium that is accumulated during growth in tilapia and goldfish is obtained from the water (Flik *et al.*, 1984c; Berg, 1968, 1970; Mugiya & Ichii, 1981; Ichii & Mugiya, 1983). The bulk of this uptake probably takes place via the chloride cells located in the branchial epithelium (Pang *et al.*, 1980; Payan *et al.*, 1981; Dunel-Erb *et al.*, 1983; Flik *et al.*, 1984d). It seems reasonable then to predict that branchial calcium uptake is an important element in the mechanism of calcium homeostasis in fish, that it must be under some control, and that at least part of this control is mediated by prolactin. This prediction is supported by the observation that ovine prolactin stimulates the high-affinity Ca^{2+}-ATPase activity in American eel branchial epithelial plasma membranes (Flik *et al.*, 1984b), and that prolactin synthesis is stimulated in tilapia exposed to low ambient levels of calcium (Wendelaar Bonga *et al.*, 1984b). Also relevant is that exposure to low ambient calcium levels stimulates calcium uptake in the killifish, *Fundulus heteroclitus* (Mayer-Gostan *et al.*, 1983), in tilapia (Flik *et al.*, 1984d) and in rainbow trout (S.F. Perry, personal communication). It should be pointed out, however, that concerning this last effect of low ambient calcium levels there are deviating observations, viz. the results of Höbe *et al.*(1984), who reported that calcium influx in rainbow trout and bullheads *(Ictalurus nebulosus)* was largely independent of ambient calcium levels.

The present study was undertaken to investigate the effects of prolactin on the kinetics of calcium exchange in growing, actively feeding tilapia. The hypothesis was that prolactin treatment would enhance net calcium uptake in these fish and that the treated fish would be able to deposit Ca in their bone more efficaciously. We analyzed the effect of ovine prolactin on both influx and efflux of calcium in freshwater male tilapia. In addition, the internal distribution of the Ca^{2+} taken up was traced in order to determine the effect of prolactin on calcium compartmentalization in the fish and to obtain an estimate of the readily-exchangeable calcium pools.

MATERIALS AND METHODS

Male tilapia, *Oreochromis mossambicus*, were obtained from laboratory stock, kept at 28°C in Nijmegen tap water under conditions as described earlier (Flik *et al.*, 1984c). The calcium concentration in the water was 0.8 mM in all cases. The fish were fed and continued to grow throughout the experiments.

All chemicals used were purchased from Sigma and were reagent grade. Ovine

prolactin (specific activity 31.5 IU/mg protein) was a generous gift of the National Institutes of Health (Hormonal Division, Endocrinological Department) Bethesda, USA. The radiotracers $^{45}Ca^{2+}$ and $^{47}Ca^{2+}$ were purchased (Amersham International plc, U.K.) as $CaCl_2$ in aqueous solution. Specific activities were 9.25-37.5 GBq/mol Ca and >0.74 GBq/mol Ca for ^{45}Ca and ^{47}Ca, respectively.

Ca determinations

Plasma total Ca was determined by atomic absorption spectrophotometry or by the use of a commercial Ca kit (Sigma) as described previously (Flik *et al.*, 1984c). Tissue total Ca contents were estimated using a thymol-blue colorimetric method (Gindler & King, 1972), after digesting the tissue in concentrated HNO_3. Radiotracer activities were determined with a gamma-ray spectrophotometer (^{47}Ca) or by liquid scintillation counting (^{45}Ca).

Ca^{2+}-flux determinations

Unidirectional Ca^{2+}-fluxes between intact fish and the water were determined and calculated as described in detail earlier (Flik *et al.*, 1984c). Total Ca^{2+}-influx rates were calculated from the slopes of apparently linear whole-body $^{47}Ca^{2+}$-accumulation curves and the water specific activity using a whole-body counter (Fig. 2a). Both total and branchial efflux rates of Ca^{2+} from the fish were determined. Total efflux rates were calculated on the basis of apparently constant whole-body $^{47}Ca^{2+}$ losses, using plasma Ca specific activities (Fig. 2b). Branchial efflux rates of Ca^{2+} were determined four days after intraperitoneal injection of $^{45}Ca^{2+}$ on the basis of apparently linear curves for tracer appearance in the water and plasma ^{45}Ca specific activities. In the latter type of efflux determinations the fish were not fed for one day prior to the experiment and the urinary bladder was emptied just prior to the experiment to exclude urinary and intestinal calcium excretion during the flux measurements (Flik *et al.*, 1984c). To assess whether the injection procedure affected the flux rates, measured flux rates in the control fish were compared with calculated flux rates for the same fish on the basis of the relationships for flux rates and body weight reported earlier (Flik *et al.*, 1984c); these were: influx, $F_{fw} = 50W^{0.805}$ nmol/h Ca^{2+} and efflux, $F_{wf} = 30W^{0.563}$ nmol/h Ca^{2+}.

Hormone administration

Prolactin was dissolved in 50 mM HCl and was injected intraperitoneally with a 26-gauge needle fixed to a Hamilton® precision syringe. The dosage was 0.3 IU/g fish per 48 h; the injected volume was 50 µl maximally. Control fish received equal volumes of solvent only. Injections were given at fixed intervals. Ca^{2+}-flux determinations were performed after a minimum of 3 hormone injections and always in the mornings of the day following the last injection. In the $^{45}Ca^{2+}$-efflux experiments the second injection consisted of a single combined injection of prolactin and tracer. The experimental protocol for the different experiments is presented in Fig. 1.

Calculations, statistics and notations

To compare flux rates of groups of fish with significantly different body weights (W_i) individual flux rates were converted to flux rates related to the mean body weight of the pertinent groups (W_m), taking into account the power relations for the respective fluxes and the body weights: $F(W_i) = aW_i^b \rightarrow F(W_m) = aW_m^b = F(W_i).(W_m/W_i)^b$.

Student's t-test for unpaired observations was applied to assess statistical significance of differences between mean values. A P-value of ≤ 0.05 was taken as significant. Linear regression analysis was performed according to the least-squares method. The symbols, definitions and units were taken from Shipley and Clark (1972).

RESULTS

Tracer uptake and tracer retention

As shown in Fig. 2a, tracer uptake is significantly stimulated in prolactin treated fish. ^{47}Ca-retention (Fig. 2b) after tracer loading from the water, was not affected by prolactin treatment.

Ca^{2+}-fluxes

(Table I). Prolactin treatment significantly increased Ca^{2+}-influx with 38%. Measured influx rates of Ca^{2+} in the control group did not differ significantly from calculated Ca^{2+}-influx rates, which indicates that the handling and injection procedure did not affect influx rate. Branchial Ca^{2+}-efflux rates were 39%

Figure 1. Protocol of the experiments.

A. Determination of Ca^{2+}-influx; three intraperitoneal injections of prolactin or solvent were given on alternate days (arrows); at t=0, fish were exposed to $^{47}Ca^{2+}$-containing water for 3 h (dark bar) and the uptake was determined.

B. $^{47}Ca^{2+}$-exposure and tissue analyses; fish were exposed for 72 h to $^{47}Ca^{2+}$-containing water (dark bar) ; the last of the three hormone or solvent injections were given 24 h after the start of exposure to $^{47}Ca^{2+}$; tissue analyses were made at t=72 h.

C. Determination of branchial Ca^{2+}-efflux and tissue analyses; the injection regimen as indicated in A was followd; the second injection consisted of a cocktail of hormone (or solvent) and $^{45}Ca^{2+}$ (*); efflux determination lasted for 6-8 h and was started at t=0 (open bar); samples for tissue analyses were taken at 80 ± 3 h after tracer injection.

D. Determination of total Ca^{2+}-efflux; fish were loaded with tracer by 72-h exposure to $^{47}Ca^{2+}$-containing water (dark bar); five hormone injections were given: two prior to and one during tracer loading, one at the start of "unloading" and one 48 h afterwards; efflux rates were calculated on the basis of whole-body tracer loss during unloading (open bar)(Fig. 2b) and plasma ^{47}Ca specific activities.

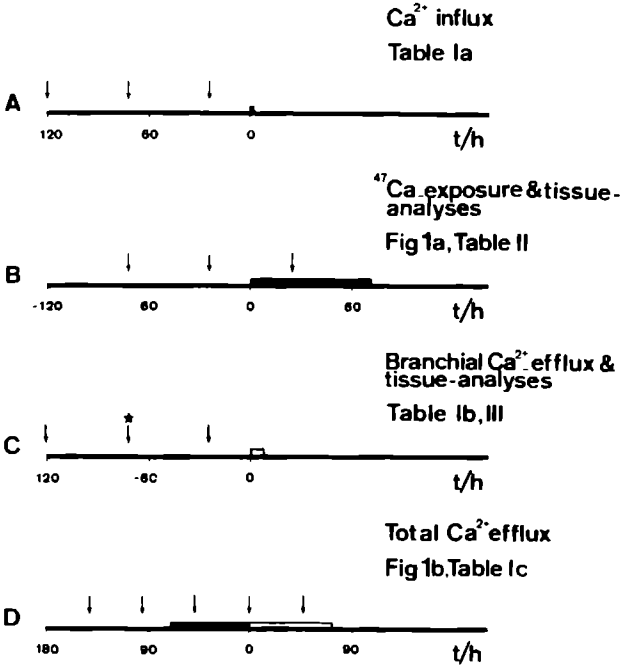
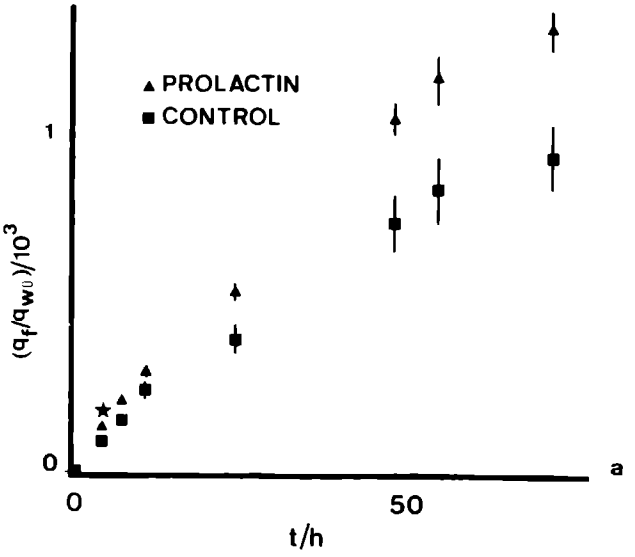
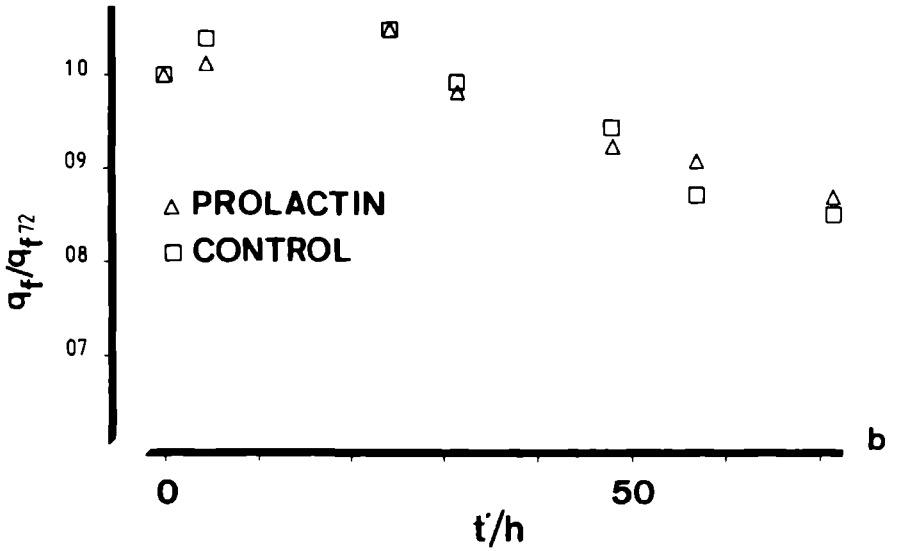


Figure 2. (a) Whole-body $^{47}Ca^{2+}$-uptake from the water in prolactin- and solvent-treated tilapia kept in fresh water. Whole-body tracer content is presented as a fraction of the water total tracer content at t=0. Mean values for 9 fish ± S.E. are given; ★P < 0.05. (b) Whole-body tracer retention in prolactin and solvent treated tilapia after tracer-loading from the water. Whole-body tracer content at time t' is expressed as a fraction of the total amount of tracer in the body at t'_0 ($t'_0 = t_{72}$ of Fig. 1a). Whole-body Ca^{2+}-efflux rates were calculated from the interpolated mean plasma ^{47}Ca specific activities at 62 h and the whole-body tracer loss over the time period t' = 52 - 72 h. Of each experimental group 4 fish were killed at t' = 52 h and 5 fish at t' = 72 h.





lower in the prolactin treated fish than in the control fish. Branchial efflux rates in the control fish did not differ significantly from calculated efflux rates in these fish, which we have taken as evidence that the procedure itself did not noticeably influence the flux rates. Total body efflux rates of Ca^{2+} were reduced by 55% in prolactin treated fish. On a percentage basis then, the average net uptake rate of Ca^{2+} from the water, $F_{net} = F_{fw} - F_{wf}$, is increased by 38 - (-39) = 77%. If we consider the effect of prolactin on the net uptake of Ca^{2+} from the water as the difference between its effect on influx (F_{fw}) and that on total efflux ($F_{wf}(t)$), the overall effect comes to 38 - (-55) = 93% increased net uptake of Ca^{2+} in prolactin treated tilapia. In all cases the prolactin treated groups of tilapia showed significant increases in plasma Ca (Table I and III).

Table I. Effects of ovine prolactin on Ca^{2+}-flux rates in freshwater tilapia.

Body weights, plasma Ca and flux rates are given for three experiments (A, B, C) for the determination of whole-body influx rates, branchial efflux rates and total efflux rates, respectively. Measured flux rates in control fish were compared with calculated flux rates, which are represented by the values in parentheses. Mean values ± S.E. are given, with n=9, n=10 and n=9 for A, B and C respectively.

A: Whole-body Ca^{2+}-influx rates

	Controls	Prolactin-treated	%Change	P
Fish weight (g)	10.7 ± 1.29	10.7 ± 1.29	-	*n.s.*
Influx, F_{fw} (nmol/h)	411 ± 128 (337 ± 41)	517 ± 148	+38	0.05
Plasma Ca (mM)	2.82 ± 0.17	3.15 ± 0.18	+12	0.001

B: Integumental Ca^{2+}-efflux rates

	Controls	Prolactin-treated	%Change	P
Fish weight (g)	13.21± 0.20	13.19± 0.25	-	*n.s.*
Efflux, F_{wf} (nmol/h)	149 ± 49	92 ± 44	-39	0.02
Plasma Ca (mM)	2.85 ± 0.13	3.16 ± 0.12	+11	0.001

C: Total Ca^{2+}-efflux rates

	Controls	Prolactin-treated	%Change	P
Fish weight (g)	10.57± 1.25	11.33± 1.32	-	*n.s.*
Efflux, $F_{wf}(t)$ (nmol/h)	178 ± 54	115 ± 16	-55	0.01
Plasma Ca (mM)	2.89 ± 0.14	3.11 ± 0.11	+ 8	0.001

Tissue Ca analyses

(Table II and III). Prolactin treatment significantly increased ^{47}Ca specific activities of vertebral and scalar bone, but plasma ^{47}Ca specific activities of prolactin treated fish, although somewhat higher were not significantly different from those of control fish (values were: 354 ± 49 and 385 ± 52 cpm/µmol Ca for controls and prolactin treated fish, respectively). SA_r-values for vertebrae and scales in prolactin treated fish increased by 17% and 19%, respectively.

Table III lists the effects of prolactin treatment on the Ca content and the relative ^{45}Ca specific activities of plasma and the three bony compartment. The SA_r-values, representing the readily exchangeable Ca-pool of the tissue, were not affected by prolactin treatment. However, skeletal, dermal and scalar bone showed significant increases in Ca-content in the prolactin treated fish.

Table II. Effects of ovine prolactin on tissue ^{47}Ca specific activity after 72 h exposure of fish to $^{47}Ca^{2+}$-containing water.

SA_r-values ($SA_r = 100 \times SA_t/SA_p$) represent tissue specific activities (SA_t) relative to plasma specific activities (SA_p). Mean values for five fish are given ± S.E.

	SA_r			
	Controls	Prolactin-treated	%Change	P
Plasma	100	100	-	*n.s.*
Vertebrae	6.37 ± 0.37	7.45 ± 0.38	17	0.02
Scales	7.38 ± 0.47	8.80 ± 0.41	19	0.01

Table III. Effects of ovine prolactin on tissue Ca contents and SA_r-values.

Plasma Ca is expressed in mM. Bone tissue Ca contents is expressed in mmol/g dry weight tissue. Mean values for 10 fish ± S.E. are given.

	Ca-content		SA_r	
	Controls	Prolactin-treated	Controls	Prolactin-treated
Plasma	2.85 ± 0.13	3.16 ± 0.12†	100	100
Vertebrae	5.20 ± 0.69	5.83 ± 0.69†††	16.9 ± 9.9	18.6 ± 11.8
Operculum	5.93 ± 0.37	6.75 ± 0.83††	16.2 ± 7.1	16.9 ± 9.1
Scales	4.83 ± 0.38	5.41 ± 0.66†	26.9 ± 7.6	24.2 ± 11.7

†: $P < 0.001$; ††: $P < 0.02$; †††: $P < 0.05$

DISCUSSION

Prolactin-induced hypercalcemia

Ovine prolactin produced a state of frank hypercalcemia in fresh water adapted tilapia. This confirms earlier reports concerning tilapia (Wendelaar Bonga & Flik, 1982) and other teleosts as sticklebacks, *Gasterosteus aculeatus* (Wendelaar Bonga & Flik, 1982; Wendelaar Bonga & Greven, 1978), American eels, *Anguilla rostrata*

(Copp *et al.*, 1981; Flik *et al.*, 1984b), rainbow trout, *Salmo gairdneri* (Ma & Copp, 1981) and the killifish, *Fundulus heteroclitus* (Pang *et al.*, 1981). We believe that the observed effect was a physiological rather than a pharmacological response to prolactin, because the fish used in the present study were held in water with a calcium concentration of 0.8 mM at which concentration the endogenous production of prolactin is submaximal (Wendelaar Bonga *et al.*, 1984b). Also, in tilapia kept under identical conditions, homologous prolactin can cause hypercalcemia, as judged by the effect of ectopically implanted prolactin lobes (Wendelaar Bonga *et al.*, 1984a). We conclude, therefore, that prolactin is a naturally occurring hypercalcemic hormone in teleosts. As the degree of bone mineralization was also increased, the prolactin induced stimulation of Ca^{2+}-influx coupled with the reduced Ca^{2+}-efflux caused this hypercalcemia.

Prolactin and Ca^{2+}-fluxes

Our measurements of Ca^{2+}-influx represent whole-body influx. However, we advanced arguments that the bulk of total influx takes place across the gills (Flik *et al.*, 1984c) and that, therefore, in freshwater tilapia whole-body influx rates in fact can be equated with branchial influx rates. The data presented in this paper give further evidence for this thesis. The prolactin induced hypercalcemia in freshwater tilapia is accompanied by a stimulation of Ca^{2+}-influx. This observation corroborates our report on prolactin induced hypercalcemia in freshwater American eels, that was accompanied by a stimulation of high-affinity Ca^{2+}-ATPase activity in the gills (Flik *et al.*, 1984b). A similar enzyme activity was demonstrated in the gills of tilapia, and, moreover, this enzyme activity was shown to be directly related to active Ca^{2+}-transport in gill plasma membranes (Flik *et al.*, 1984e). It seems reasonable then to state that prolactin stimulates active transport mechanisms in the gills and thereby promotes Ca^{2+}-influx. We have reported evidence that the branchial Ca^{2+}-uptake mechanisms are located in the chloride cells (Flik *et al.*, 1984d). Moreover, our unpublished measurements of chloride cell numbers in the branchial area show that after injection of ovine prolactin or after implantation of tilapia prolactin cell implants chloride cell densities are increased in these fish. Thus, the stimulatory action of prolactin on whole-body Ca^{2+}-influx rates reported above, therefore, may have resulted from both a prolactin-induced increase in Ca^{2+}-transport mechanisms in the chloride cells and a proliferation of the chloride cells in the gills. Whether the stimulatory effect of prolactin on branchial Ca^{2+}-uptake mechanisms is direct or indirect, e.g. through the action of steroid hormones, requires further investigation. Fleming

and coworkers (1971) have given evidence that in *Fundulus kansae* prolactin stimulates the production of cortisol, the major mineralocorticoid in fish. It has been suggested that branchial chloride cell densities in tilapia are positively correlated with circulating cortisol levels (Foskett *et al.*, 1981).

In fish residing in hypocalcic fresh waters, Ca^{2+}-efflux will result from passive diffusion of Ca^{2+} over the integument and from urinary and intestinal outflow of Ca. As discussed earlier (Flik *et al.*, 1984c), integumental Ca^{2+}-efflux essentially equals branchial efflux of Ca^{2+}. Data on total body Ca^{2+}-efflux and integumental Ca^{2+}-efflux, then, allow discrimination between branchial and extrabranchial efflux rates. Branchial and total body efflux rates determined in the present study for control fish agree well with those reported previously for untreated tilapia kept under identical conditions (Flik *et al.*, 1984b, 1984c). Prolactin treatment decreased total body and branchial efflux rates by 55% and 39%, respectively. This implies that also the extrabranchial efflux routes are affected by prolactin treatment. The extrabranchial routes mainly concern intestine and urinary tract. Our approach in determining Ca^{2+}-efflux rates does not allow any distinction between the contributions of these organs to Ca^{2+}-transport. Both may be implicated, however, for it has been shown that prolactin exerts osmoregulatory effects not only on the gills but also on the intestine (Morley *et al.*, 1981), kidney (Forster, 1975) and urinary bladder (Doneen, 1981) of freshwater fish. From our data it may be concluded that, although the effects of prolactin on Ca^{2+}-fluxes may extend to extrabranchial sites, the gills certainly are the most important target for this hormone.

Our Ca^{2+}-efflux data implicate that integumental permeability to Ca^{2+} is reduced by prolactin. That this permeability to Ca^{2+} is determined by the constitution and numbers of the paracellular pathways of the epithelium was concluded earlier (Flik *et al.*, 1984c). In the same species the significance of the paracellular routes for permeability to ions was first shown for Na^{+}-efflux by Dharmamba & Maetz (1972). Prolactin decreases branchial permeability to water and ions in tilapia (Dharmamba & Maetz, 1972; Wendelaar Bonga & Van der Meij, 1980, 1981), in freshwater Japanese as well as European eels (Ogawa, 1977; Ogasaware & Hirano, 1984; Maetz *et al.*, 1967a), the killifish, *Fundulus heteroclitus* (Maetz *et al.*, 1967b) and the green molly, *Poecilia latipinna* (Ensor & Ball, 1968). We now suggest that the control of integumental permeability to Ca^{2+} is the pivotal event in prolactin action on hydromineral regulation, for it has been shown earlier that the interrelationship between endogenous prolactin and ambient Ca^{2+} underlies the control of branchial permeability to water and ions (Wendelaar Bonga & Van der Meij, 1980, 1981; Ogasawara & Hirano, 1984).

Prolactin and internal calcium reservoirs

The hypercalcemia and increasing Ca-content of the bony tissues, following prolactin stimulated Ca^{2+}-uptake in tilapia, shows that in this species plasma calcium freely exchanges with the calcium in the bone and can be stored there. It was shown earlier that during acclimation to low-calcium water tilapia demineralizes its bone (Flik *et al.*, 1984d). From the present results it may be concluded that "excess" Ca^{2+} in the plasma leads to enhanced mineralization. Clearly then, the acellular bone of tilapia is intimately associated with calcium metabolism, acting as an internal compartment which allows for storage as well as mobilization of Ca^{2+}.

Although in this study no separate determination of free and protein-bound calcium was carried out, it has been shown that in tilapia prolactin-induced hypercalcemia is accompanied by elevated levels of free calcium (Wendelaar Bonga *et al.*, 1983). It does not seem to be too imprudent then to state that it was the free Ca^{2+} in the blood, increased by the prolactin treatment, that was deposited into the bony tissues. This situation in tilapia resembles the calcium exchange process in otoliths of rainbow trout, reported by Mugiya (1984). Mugiya showed that the degree of calcium deposition in these otoliths was positively correlated with total plasma calcium levels. From his *in vitro* studies he concluded that the calcium exchange between the otoliths and the endolymph parallels the fluctuations in free Ca^{2+}-levels in the endolymph.

In our study, the effect of prolactin on the bony tissue was restricted to an analysis of the bone Ca-contents. It is relevant to mention that is has earlier been demonstrated that prolactin treatment of tilapia does not affect the lining osteoblasts of its bone, at least judged on the basis of the ultrastructural features of this tissue (Wendelaar Bonga & Flik, 1982). The observed effect of prolactin on the fish bone, therefore, seems indirect and is different from that exerted by growth hormone on bony tissue in mammals. In the latter case the hormone stimulates the activity of the lining osteoblasts, which, in turn, results in growth of bone. However, that in fish also the lining cells are involved in controlling bone development is evident from studies concerning effects of other hormones. E.g. in tilapia calcitonin as well as 24,25-dihydroxyvitamin D_3 exert their effect on bone through activation of the lining cells (Wendelaar Bonga & Lammers, 1982; Wendelaar Bonga *et al.*, 1983a).

The fact that the SA_r-values for bones in the controls and the prolactin treated tilapia are very similar indicates that the increase in bone calcium content is not noticeably accompanied by a relative increase in the readily-exchangeable Ca-pool of the bone. This then further supports our conclusion that true storage of Ca in the bones occurs, when tilapia are treated with prolactin.

In conclusion, the results presented in this paper confirm that prolactin is a hypercalcemic hormone in freshwater teleosts and show that in tilapia the hypercalcemic effect of prolactin results from mainly a dual action, viz. stimulation of branchial Ca^{2+}-uptake and reduction of integumental permeability to Ca^{2+}. In terrestrial vertebrates calcium homeostasis depends on the hypercalcemic parathormone that stimulates cell-mediated Ca-resorption from the bone. In fish, calcium metabolism differs essentially from that in land-living vertebrates, and it seems that calcium homeostasis depends on the governing hypercalcemic hormone in these aquatic vertebrates: prolactin.

REFERENCES

BERG, A. (1968) Studies on the metabolism of calcium and strontium in freshwater fish.-I. Relative contribution of direct and intestinal absorption. Me.Inst. Ital.Idrobiol. 23: 161-196.

BERG, A. (1970) Studies on the metabolism of calcium and strontium in freshwater fish.-II. Relative contribution of direct and intestinal absorption in growth conditions. Me.Inst.Ital.Idrobiol. 26: 241-255.

COPP, D.H., MA, S.W.Y., DATTA, A., SIM, M. & SZETO, K. (1982) Symposium proceedings of the 9th International Symposium on Comparative Endocrinology (Hong Kong, 1981) Hong Kong University Press. In Press.

DHARMAMBA, M. & MAETZ, J. (1972) Effects of hypophysectomy and prolactin on the sodium balance of *Tilapia mossambica* in fresh water. Gen.Comp.Endocrinol. 19: 175-183.

DONEEN, B.A. (1976) Biological activities of mammalian and teleostean prolactins and growth hormones on mouse mammary gland and teleost urinary bladder. Gen. Comp.Endocrinol. 30: 34-42.

DUNEL-ERB, S., HÖBE, H. & LAURENT, P. (1983) Chloride cell proliferation in soft water living trout. L Proceedings Symp.Eur. de Physiol.Comp., Strasbourg.

ENSOR, D.M. & BALL, J.N. (1968) Prolactin and freshwater sodium fluxes in *Poecilia latipinna* (Teleostei). J.Endocrinol. 41: xvi.

FLEMING, W.R., BALL, J.N. & CONAWAY, C.H. (1971) The effects of a saline environment and ACTH on the interrenal of *Fundulus kansae*. Z.Vergl.Physiol. 74: 121-126.

FLIK, G., WENDELAAR BONGA, S.E. & FENWICK, J.C. (1984a) Ca^{2+}-dependent phosphatase and Ca^{2+}-dependent ATPase activities in plasma membranes of eel gill epithelium. -II. Evidence for transport high-affinity Ca^{2+}-ATPase. Comp.Biochem.Physiol. In Press.

FLIK, G., WENDELAAR BONGA, S.E. & FENWICK, J.C. (1984b) Ca^{2+}-dependent phosphatase and Ca^{2+}-ATPase activities in plasma membranes of eel gill epithelium.-III. Stimulation of branchial high-affinity Ca^{2+}-ATPase during prolactin induced hypercalcemia. Comp.Biochem.Physiol. In Press.

FLIK, G., FENWICK, J.C., KOLAR, Z., MAYER-GOSTAN, N. & WENDELAAR BONGA, S.E. (1984c) Whole-body Ca^{2+}-flux rates in the cichlid teleost fish *Oreochromis mossambicus*, adapted to fresh water. Am.J.Physiol. submitted.

FLIK, G., FENWICK, J.C., KOLAR, Z., MAYER-GOSTAN, N. & WENDELAAR BONGA, S.E. (1984d) Effects of low ambient calcium levels on whole-body Ca^{2+}-flux rates and internal calcium pools in the freshwater cichlid teleost fish *Oreochromis mossambicus*. Am.J.Physiol. submitted.

FORSTER, R.C. (1975) Changes in urinary bladder and kidney function in the starry flounder *(Platichthys stellatus)* in response to prolactin and fresh water transfer. Gen.Comp.Endocrinol.

FOSKETT, J.K., LOGSDON, G.D., TURNER, T., MACHEN, T.E. & BERN, H.A. (1981) Differentiation of the chloride extrusion mechanism during seawater adaptation of a teleost fish, the cichlid *Sarotherodon mossambicus*. J.exp.Biol. 93: 209-224.

HÕBE, H., LAURENT, P. & McMAHON, B.R. (1984) Whole-body calcium flux rates in freshwater teleosts as a function of ambient calcium and pH levels: a comparison between the euryhaline trout, *Salmo gairdneri*, and the stenohaline bullhead, *Ictalurus nebulosus*. J.exp.Biol.: In Press.

ICHII, T. & MUGIYA, Y. (1983) Effects of a dietary deficiency in calcium on growth and calcium uptake from the aquatic environment in the goldfish, *Carassius auratus*. Comp.Biochem.Physiol. 74A: 259-262.

MA, S.W.Y. & COPP, D.H. (1981) Prolactin and calcium metabolism in teleosts. In: Hormonal control of Calcium metabolism. (Cohn, D.V., Talmage, R.V. and Matthews, J.L. eds) Excerpta Medica, Amsterdam: 425.

MAETZ, J., SAWYER, W.H., PICKFORD, G.E. & MAYER, N. (1967a) Evolution de la balance minérale de sodium chez *Fundulus heteroclitus* au cours du transfert d'eau de mer en eau douce: effets de l'hypophysectomie et de la prolactine. Gen.Comp. Endocrinol. 8: 163-176.

MAETZ, J., MAYER, N. & CHARTIER-BARADUC, M.M. (1967b) La balance minérale du sodium chez *Anguilla anguilla* en eau de mer en eau douce et au cours de transfert d'un milieu à l'autre: effets de l'hypophysectomie et de la prolactine. Gen.Comp.Endocrinol. 8: 177-188.

MAYER-GOSTAN, N., BORNANCIN, M., DeRENZIS, G., NAON, R., YEE, J.A., SHEW, R.L. & PANG, P.K.T. (1983) Extraintestinal calcium uptake in the killifish, *Fundulus heteroclitus*. J.exp.Zool. 227: 329-338.

MORLEY, M., CHADWICK, A. & TOUNSY, E.M. (1981) The effect of prolactin on water absorption by the intestine of the trout *(Salmo gairdneri)*. Gen.Comp.Endocrinol. 44: 64-68.

MUGIYA, Y. (1984) Diurnal rhythm in otolith formation in the rainbow trout, *Salmo gairdneri:* seasonal reversal of the rhythm in relation to plasma calcium concentrations. Comp.Bioch.Physiol. 78A: 289-293.

OGASAWARA, T. & HIRANO, T. (1984) Effects of prolactin and environmental calcium on osmotic water permeability of the gills in the eel, *Anguilla japonica*. Gen.Comp.Endocrinol. 53: 315-325.

OGAWA, M. (1977) The effect of hypophysectomy and prolactin treatment on the osmotic water influx into the isolated gills of the Japanese eel *(Anguilla japonica)* Canada. J.Zool. 55: 872-876.

PANG, P.K.T., SCHREIBMAN, M.P. & GRIFFITH, R.W. (1973) Pituitary regulation of serum calcium levels in the killifish, *Fundulus heteroclitus* L. Gen.Comp. Endocrinol. 21: 536-542.

PANG, P.K.T., SCHREIBMAN, M.P., BALBONTIN, F. & PANG, R.K. (1978) Prolactin and pituitary control of calcium regulation in the killifish, *Fundulus heteroclitus*. Gen.Comp.Endocrinol. 36: 306-316.

PANG, P.K.T., GRIFFITH, R.W., MAETZ, J. & PIC, P. (1980) Calcium uptake in fishes. In: Epithelial transport in the lower vertebrates (ed. B. Lahlou), Cambridge University Press, Cambridge: 121-132.

PANG, P.K.T. (1981) Hypercalcemic effects of ovine prolactin on intact killifish, *Fundulus heteroclitus*, subjected to different environmental calcium-challenges. Gen.Comp.Endocrinol. 43: 252-255.

PAYAN, P., MAYER-GOSTAN, N. & PANG, P.K.T. (1981) Site of calcium uptake in the freshwater trout gill. J.exp.Zool. 216: 345-347.

SHIPLEY, R.A. & CLARK, R.E. (1972) Tracer methods for *in vivo* kinetics-theory and applications. New York and London, Academic press.

SIMKISS, K. (1974) Calcium metabolism of fish in relation to ageing. In: Ageing of fish (ed. Begenal, T.B.), Unwin Brothers, Old Woking: 1-12.

WENDELAAR BONGA, S.E. & GREVEN, J.A.A. (1978) The relationship between prolactin cell activity, environmental calcium, and plasma calcium in the teleost *Gasterosteus aculeatus*. Observations on stanniectomized fish. Gen.Comp.Endocrinol. 36: 90-101.

WENDELAAR BONGA, S.E. & VAN DER MEIJ, J.C.A. (1980) The effect of ambient calcium on prolactin cell activity and plasma electrolytes in *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. 40: 391-401.

WENDELAAR BONGA, S.E. & VAN DER MEIJ, J.C.A. (1981) Effect of ambient osmolarity and calcium on prolactin cell activity and osmotic water permeability of the gills in the teleost *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. 43: 432-442.

WENDELAAR BONGA, S.E. & FLIK, G. (1982) Prolactin and calcium metabolism in a teleost fish. In: Comparative endocrinology of calcium regulation (Oguro, C. and Pang, P.K.T. eds) Tokyo, Japan Scientific Societies Press: 21-26.

WENDELAAR BONGA, S.E. & LAMMERS, P.I. (1982) Effects of calcitonin on ultrastructure and mineral content of bone and scales of the cichlid teleost *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. 48: 60-70.

WENDELAAR BONGA, S.E., LAMMERS, P.I. & VAN DER MEIJ, J.C.A. (1983a) Effects of 1,25- and 24,25-dihydroxyvitamin D_3 on bone formation in the cichlid teleost *Sarotherodon mossambicus*. Cell Tissue Res. 228: 117-126.

WENDELAAR BONGA, S.E., LOEWIK, C.W.G.M. & VAN DER MEIJ, J.C.A. (1983b) Effects of external Mg^{2+} on branchial osmotic water permeability and prolactin secretion in the teleost *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. 52: 222-231.

WENDELAAR BONGA, S.E., FLIK, G. & FENWICK, J.C. (1984a) Prolactin and calcium metabolism in fish: effects on plasma calcium and high-affinity Ca^{2+}-ATPase in gills. In: Endocrine control of bone and calcium metabolism (D.V. Cohn, J.T. Potts Jr. and J. Fujita eds) Elsevier Science Publishers B.V.: 188-190.

WENDELAAR BONGA, S.E., FLIK, G., LOEWIK, C.W.G.M. & VAN EYS, G.J.J.M. (1984b) Environmental control of prolactin secretion in the teleost fish *Oreochromis mossambicus*. Comp.Endocrinol. In Press.

CHAPTER IX

GENERAL DISCUSSION

It is well established now that freshwater fish show calcium homeostasis in the body fluids and exhibit a remarkable capacity to extract Ca^{2+} from the water. Also, it is generally accepted that the gills are the primary site for the uptake of calcium from the water. The purpose of the investigations presented in this thesis was to fill the gaps in our knowledge concerning the mechanisms underlying the uptake of Ca^{2+} from the water, the kinetics of such Ca^{2+}-uptake processes and the way hormonal control is exerted. Using American eel and tilapia, for the first time the existence of a Ca^{2+}-transporting mechanism in plasma membranes of fish branchial epithelium was demonstrated. The involvement of this mechanism in transepithelial Ca^{2+}-transport was made plausible by comparing in tilapia the *in vitro* Ca^{2+}-transport capacity of the gills with the *in vivo* influx rates of Ca^{2+} through the gills. The hormone prolactin was shown to be involved in controlling Ca^{2+}-exchange with the water, both the influx (representing active Ca^{2+}-transport) and the efflux (depending on integumental permeability to Ca^{2+}). In tilapia bony tissue plays a role in calcium metabolism acting as an internal Ca^{2+}-buffer system and as a mineral store. The control of Ca^{2+}-exchange with the water, however, seems of primary importance for calcium homeostasis.

Mechanisms for Ca^{2+}-transport in branchial epithelium

The epithelium covering the gills of freshwater fish is a typical "tight" epithelium. The working hypothesis in our studies on Ca^{2+}-transport mechanisms in this epithelium, therefore, was that the transepithelial Ca^{2+}-influx follows a transcellular route. Assuming analogy to the situation for transcellular Ca^{2+}-transport in e.g. rat intestinal mucosa as described by Van Os & Ghijsen (1981), it was postulated that a Ca^{2+}-translocating ATPase in the basolateral plasma membranes of the branchial epithelial cells energizes Ca^{2+}-extrusion to the blood.

First, a procedure was developed for the preparation of plasma membrane-enriched fractions from branchial epithelium, applying more rigorous criteria for "purity" of membrane fractions than are usually reported (e.g. Ma & Copp, 1974; Ho & Chan, 1980). Especially important in this respect was the use of Na^+/K^+-ATPase activity, determined as the K^+-dependent, ouabain-sensitive Na^+-ATPase activity,

as a plasma membrane marker. This choice was made for the following reasons:
- Na^+/K^+-ATPase activity exhibits membrane specificity; alkaline phosphatase activity used as plasma membrane marker by others (Ma & Copp, 1974) is of ubiquitous subcellular origin and, moreover, heterogeneous; in our procedure, this last enzyme activity was not purified along with Na^+/K^+-ATPase activity;
- Na^+/K^+-ATPase is an extrinsic enzyme; the effects of detergents on membrane-bound Na^+/K^+-ATPase were tested to assess conditions of optimal substrate accessibility in membrane vesicle preparations;
- the majority of the Na^+/K^+-ATPase in the gills is concentrated in the chloride cells (Hootman & Philpott, 1978, 1979; Karnaky, 1980); these cells represent the ion-transporting units of the branchial epithelium and Na^+/K^+-rich membrane fractions should predominantly originate, then, from these particular cells.

Membrane fractions, highly enriched in Na^+/K^+-ATPase activity, hydrolyze ATP upon addition of Ca^{2+}. This Ca^{2+}-*activated* ATPase activity has formerly been presumed to be the enzymic basis for active transport of Ca^{2+} through the gills (e.g. Ho & Chan, 1980; Shepard, 1981). However, our studies allowed to identify this enzymic activity as a heterogeneous, *non-specific* phosphatase activity.

The demonstration of transport Ca^{2+}-ATPase activity in basolateral plasma membranes of rat enterocytes by Ghijsen & Van Os (1979) and by Ghijsen *et al.* (1980), gave the impetus to our discovery of a very similar enzyme activity in plasma membrane fractions of fish gills. The know-how and experience of Van Os, Ghijsen and co-workers was gratefully applied in our studies. Ca^{2+}-*stimulated* Mg∿ATP hydrolysis by eel gill plasma membranes appeared to result from a high-affinity Ca^{2+}-ATPase and a low-affinity phosphatase activity. Only the characteristics of the high-affinity Ca^{2+}-ATPase activity met the criteria for a Ca^{2+}-transporting ATPase. Similar, multiple Ca^{2+}-stimulated ATPase activity has been demonstrated in plasma membranes of rate enterocytes (Ghijsen & Van Os, 1980), of hen oviduct shell gland (Coty & McConkey, 1982), of pancreatic islet cells (Pershadsing *et al.*, 1980) and of Ehrlich ascites tumor cells (Klaven *et al.*, 1983) and, thus, such complex enzyme activity seems to occur widely.

From the simultaneous occurrence of Ca^{2+}- or Mg^{2+}-activated ATPases and Ca^{2+}-stimulated ATPases in plasma membranes it may be inferred that to demonstrate transport Ca^{2+}-ATPase hydrolytic activities in plasma membranes, the use of assay-media containing Ca^{2+}-buffers with Mg∿ATP as a substrate is mandatory. Such criteria invalidate the claim by Doneen (1981) of the demonstration of transport Ca^{2+}-ATPase activity in the gills of *Gillichthys mirabilis*. Also, since the existence of transport Ca^{2+}-ATPase can be deduced only by differentiating it from a heterogeneous pool of phosphatase activities, it follow that the direct and specific

cytochemical demonstration of transport Ca^{2+}-ATPase activity via Wachstein-Meisel procedures (Ando *et al.*, 1981) seems impossible. Therefore, cytochemical data reported in the literature on the localization of transport Ca^{2+}-ATPases should be reevaluated.

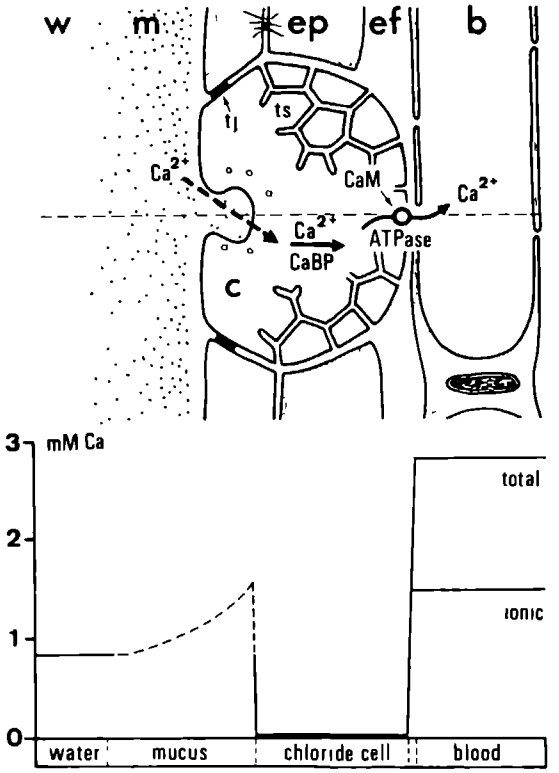
The involvement of plasma membrane-bound high-affinity Ca^{2+}-ATPase activity in transepithelial Ca^{2+}-transport in the gills is strongly supported by the demonstration of an ATP-dependent Ca^{2+}-transporting process with high-affinity for Ca^{2+} in plasma membrane vesicles. Of particular interest is that the Ca^{2+}-transporting capacity of the branchial apparatus determined *in vitro* was of the same order of magnitude as the branchial Ca^{2+}-influx rates measured *in vivo*. We consider the direct comparison of *in vivo* and *in vitro* measurements of Ca^{2+}-transport in our set-ups an essential and powerful step in assessing the physiological meaning of the data obtained.

The results and conclusions on the mechanisms of transepithelial Ca^{2+}-transport in fish gills are summarized in the model presented in Fig. 1.

Ca^{2+}-uptake from the water and the role of internal calcium stores in calcium metabolism

As a basis for the calculation of bidirectional Ca^{2+}-fluxes between water and fish, a model was chosen consisting of two compartments: the water and the extracellular fluids of the fish. To approximate steady-state conditions for calcium as close as possible, only well-acclimated fish were used, while the composition of the water was carefully monitored and kept constant. The use of ^{47}Ca and a whole-body counter enabled us to establish the behaviour of calcium tracers in intact tilapia and, subsequently, to design experimental conditions such that first-order tracer kinetics reflect Ca^{2+}-fluxes between the water and the fish. For the interpretation of our data on calcium kinetics and internal distribution of calcium it is important to realize that we consistently used activily feeding, growing tilapia. Our fish, therefore, are in a positive calcium balance which is clear, among other things, by a net influx of Ca^{2+} from the water and calcium accretion in the bone. The reasoning behind the use of fully acclimated fish was that only such fish may be predicted to give their full physiological response to changes in the ambient water or to changes in the endocrine status of the fish, that affect their calcium metabolism. These concepts underlying our Ca^{2+}-flux determinations were validated by the fact that net Ca^{2+}-influx, calculated as the difference of in- and efflux of Ca^{2+}, were in agreement with the actual calcium balance of the fish. Moreover, influx and efflux rates of Ca^{2+} -

Figure 1. Upper part: diagram of Ca^{2+}-transport across the branchial epithelium of a freshwater fish. Ca^{2+} from the water (w) enters the epithelium (ep) via chloride cells down an electrochemical gradient. Mucus (m) covering the epithelium may steepen the Ca^{2+}-gradient over the apical membrane by concentrating Ca^{2+} topically due to its Ca^{2+}-binding activity as well as by providing an unstirred layer. Ca^{2+} is buffered in the cytosol (c) by calcium-binding proteins (CaBP). At the basolateral plasma membranes Ca^{2+} is extruded to the extracellular fluid (ef). A Ca^{2+}-transporting enzyme (ATPase), which depends on activation by calmodulin (CaM), provides energy to translocate Ca^{2+} against a steep gradient; the basolateral plasma membranes extend to a well-developed system of infoldings referred to as reticular system or tubular system (ts); tj, tight junctions.
Lower part: Ca^{2+}-concentrations along the dotted line in the upper part: in the water (0.8 mM Ca^{2+}, Nijmegen city tapwater), in the mucus (the dotted line indicates that the Ca^{2+}-concentration may increase towards the apical cell membranes), in the cytosol (approximately 0.1 µM Ca^{2+}) and in the extracellular fluid and blood (ef, b; approximately 2.8 mM total Ca and 1.5 mM Ca^{2+}, respectively).



determined by the Ca^{2+}-transport activity and the permeability to Ca^{2+} of the integument, respectively - were affected in a predictable manner by experimental manipulation of the fish. For example, a reduction of ambient calcium levels causes increased integumental permeability to water and ions in tilapia (Wendelaar Bonga & Van der Meij, 1980, 1981) and results in a transient negative calcium balance. Tilapia well-acclimated to low calcium water, however, re-establish a positive calcium balance. In such fish Ca^{2+}-influx rates surpassed Ca^{2+}-efflux rates (which were enhanced compared to freshwater fish) resulting in net uptake of Ca^{2+} from the water. Prolactin-induced hypercalcemia could be traced back to an enhanced net uptake of Ca^{2+} from the water, which resulted from a simultaneous stimulation of Ca^{2+}-influx and a reduction of Ca^{2+}-efflux. These results are in complete agreement with our findings of a stimulation by prolactin of branchial transport Ca^{2+}-ATPase activity in American eels (Flik *et al.*, 1984). They, furthermore, are in concordance with a large body of evidence that prolactin reduces integumental permeability to water and monovalent ions (Dharmamba & Maetz, 1972; Wendelaar Bonga & Van der Meij, 1980, 1981; Ogasawara & Hirano, 1984).

With respect to the occurrence of elevated plasma calcium levels, an apparent discrepancy exists between the effect of low ambient calcium and that of prolactin treatment. Under low-calcium conditions, the hypercalcemia was accompanied by a decrease in bone calcium contents, whereas the prolactin-induced hypercalcemia led to an increased bone calcium content. We tentatively ascribe the persisting decrease in calcium contents of the bone in fish under low calcium conditions to a negative phosphate balance. Both an impaired absorption of phosphate from the gut - presumably coupled to an impaired Ca^{2+} absorption from the gut such as observed in other species under low calcium conditions (Rodgers, 1984) - and an enhanced integumental efflux of phosphate might have urged the fish to mobilize phosphate from its bone, or reduce the degree of mineralization of its bony tissue. For the time being, experimental data to verify this last hypothesis are lacking which stresses the need for simultaneous evaluation of the calcium and phosphate content of the pertinent compartments.

Hormonal control of calcium homeostasis

The endocrine control that underlies calcium homeostasis in aquatic and terrestrial vertebrates differs in many respects. Mammals, birds, reptiles and terrestrial amphibians possess parathyroid glands that produce parathormone (PTH), their most important hypercalcemic hormone. In many aquatic amphibians (most of the urodeles) and in fish, parathyroids are absent. On the other hand, corpuscles

of Stannius, endocrine glands morphologically associated with the kidneys, occur only in fish. The putative hormone of the corpuscles of Stannius, hypocalcin, is a proteinaceous product, which shares antigenic determinants (Milet *et al.*, 1980) and bioactivities (our unpublished observations) with mammalian PTH. Its function however, is clearly different, for in fish it acts as a hypocalcemic factor. Freshwater fish and aquatic amphibians do have efficient control-mechanisms to counteract the threat of hypocalcemia imposed by their environment. Pang and his collegues (Pang *et al.*, 1973, 1978; Pang, 1981) were among the first to show that pituitary hormones are indispensable for fish living in hypocalcic waters. Prolactin now seems to be of particular relevance, although in some species a product from the "PAS-positive cells" of the pars intermedia seems to be involved (Olivereau *et al.*, 1980, 1981). The focuss of this discussion will be on the comparison of the role of the major hypercalcemic hormones involved in the calcium homeostasis of aquatic and terrestrial vertebrates, viz. prolactin in fish and PTH in land-living tetrapods.

Pang and coworkers showed that in killifish adapted to calcium deficient sea water, hypophysectomy leads to hypocalcemia and tetanic seizures; these disturbances of calcium homeostasis could be overcome by either supplying calcium to the water or prolactin to the fish (Pang *et al.*, 1973; Pang & Yee, 1980). Hypercalcemic actions of mammalian and fish prolactins in several species of teleost fish and in certain urodeles have since been confirmed (Fenwick, 1982; Pang & Yee, 1980; Sasayama & Oguro, 1982). In terrestrial vertebrates PTH mobilizes Ca^{2+} from the bone, mainly by stimulating osteocytic osteolysis (Parfitt, 1979). Its hypercalcemic actions further include stimulation of Ca-reabsorption in the kidneys and, indirectly via stimulation of 1α,25-dihydroxyvitamin D_3-synthesis, to absorption of Ca via the gut. The mode of action of prolactin in fish is fundamentally different as is clear from the studies presented in this thesis. Freshwater fish rely on Ca^{2+} in the ambient water for Ca-homeostasis and growth, and absorb Ca^{2+} directly from the water via their gills (Berg, 1968, 1970). In freshwater tilapia prolactin stimulates the uptake of Ca^{2+} from the water via a dual action on the integument: it enhances Ca^{2+}-influx and it decreases Ca^{2+}-efflux. Since we have substantial evidence that influx of Ca^{2+} via the skin is of minor significance in the process of Ca homeostasis - a conclusion deduced from experiments with perfused tail preparations of American eel (unpublished) - the gills remain as the major site for Ca^{2+}-exchange processes between fish and water. Further support for this last conclusion is provided by our biochemical analyses of gill epithelial plasma membranes of American eels and tilapia, which led to the discovery of high-affinity Ca^{2+}-ATPase activity. It was shown that this

activity is an expression of the calcium pump of the gills, which is involved in transepithelial Ca^{2+}-transport. The hypercalcemia induced by ovine prolactin in American eels proved to be accompanied by stimulation of this branchial high-affinity Ca^{2+}-ATPase activity. In this species, prolactin has further been shown to increase the uptake of Ca^{2+} from the water in perfused isolated gills (Ma & Copp, 1981). Thus, it appears that one action of prolactin on the integument concerns stimulation of Ca^{2+}-uptake mechanisms. The second action of prolactin that contributes to enhanced uptake of Ca^{2+} from the water, reminds of the role of prolactin in controlling integumental permeability to water and monovalent ions (Dharmamba & Maetz, 1972). We conclude from our observation of inhibitory actions of prolactin on Ca^{2+}-efflux in tilapia, that the control of integumental permeability to Ca^{2+} is also prolactin-dependent. Moreover, these results suggest that prolactin exerts an action on paracellular pathways in the branchial epithelium, for Ca^{2+}-efflux is believed to be confined to such routes. Prolactin-induced extension of tight junctions has been observed in nephron-epithelium of the stickleback, *Gasterosteus aculeatus* (Wendelaar Bonga & Veenhuis, 1974) and this process may underlie the changes in permeability to water (Ogasawara & Hirano, 1984) as well as to Ca^{2+}. The more increased positive Ca-balance which results from the dual action of prolactin on integumental Ca^{2+}-exchange mechanisms, induces hypercalcemia, which subsequently leads to enhanced storage of Ca in the bone.

Whereas prolactin in freshwater fish and PTH in terrestrial vertebrates both have hypercalcemic effects, these hormones exert opposite effects with respect to the bone calcium content. The pertinent mechanism of action of these hormones is also different (Table I).

Table I. Summary of the effects of prolactin, in fish, and PTH, in terrestrial tetrapods on blood plasma and bone.

	Prolactin	PTH
Plasma Ca	+	+
Plasma P_i	-	-
Bone mineral content	+	-
Osteocytic osteolysis	=	+
Osteoclastic activity	(=)	+
Bone apposition	=	-

+ : stimulation/increase; = : no effect; - : inhibition/decrease

The effects of PTH on bone are mediated by osteocytes and osteoclasts (Parfitt, 1979). Phylogenetically advanced teleosts such as tilapia, have acellular bone, which means that bone-forming cells are confined to the periphery of the bone. Osteoclasts are scarce or even absent (Weis & Watabe, 1979; Wendelaar Bonga *et al.*, 1983). Ultrastructural evidence has been given that the prolactin-induced increases in calcium content of the bone are not associated with activation of bone cells (Wendelaar Bonga & Flik, 1982). The prolactin-induced stimulation of calcium incorporation in fish bone is, therefore, likely brought about indirectly and leads to modification of the physico-chemical exchange processes at the interphase of the extracellular fluid and the bone. Prolactin promotes the deposition of calcium and phosphate in a molar ratio of 1.6, which is characteristic for dahllite, the most important mineral in bone (McConnell, 1973). This may explain the slight hypophosphatemia we have observed frequently after prolactin treatment of tilapia. Contrastingly, the action of PTH on mammalian bone leads to increased levels of both calcium and phosphate in the blood (Parfitt, 1979). In fish, prolactin regulates the direct exchange of Ca^{2+} with that of the ambient water. In tilapia growing in fresh water, over 90% of the calcium required for growth is taken up from the water, directly via the gills; the phosphate required for growth is absorbed from the food. Terrestrial vertebrates do not have access to such a continuously available and virtually inexhaustible external calcium source. For growth calcium has to be absorbed via the gut from intermittently ingested food. The phyletic transition to land may have been accompanied by the development of PTH-dependent hypercalcemic mechanisms in cells associated with the bone, which became essential as an internal calcium buffer for calcium homeostasis. Concomitantly, another hormone, calcitonin, became the antagonist of PTH and developed into an effective hypocalcemic hormone acting by stimulating cell-mediated calcium deposition in bone matrix. In tilapia calcitonin also stimulates cell-mediated calcium deposition in the bone (Wendelaar Bonga & Lammers, 1982), but as may be anticipated from the fact that only lining cells occur in acellular bone, the hypocalcemic effects of calcitonin in such fish are small. In fish hypocalcemic mechanisms that underly calcium homeostasis primarily depend on control by products of the Stannius corpuscles, with, interestingly, also the branchial epithelium as the major target. This last statement is evidenced by our unpublished observations that branchial influx and efflux of Ca^{2+} in stanniectomized European eels are enhanced and that the amount of Stannius corpuscle tissue in these eels is directly related to the integumental surface area.

The invasion of terrestrial habitats by vertebrates and the development of bone as an internal calcium store were apparently accompanied by the evolution

of an extensive system of bone cells (enclosed osteocytes), that respond rapidly to PTH and calcitonin.

REFERENCES

ANDO, T., FUJIMOTO, K., MAYAHARA, H., MIYAJUMA, H. & OGAWA, K. (1981) A new one-step method for the histochemistry and cytochemistry of Ca^{2+}-ATPase activity. Acta Histochem.cytochem. 14(6): 705-726.

BERG, A. (1968) Studies on the metabolism of calcium and strontium in freshwater fish.-I. Relative contribution of direct and intestinal absorption. Me.Inst. Ital.Idrobiol. 23: 161-196.

BERG, A. (1970) Studies on the metabolism of calcium and strontium in freshwater fish.-II. Relative contribution of direct and intestinal absorption in growth conditions. Me.Inst.Ital.Idrobiol. 26: 241-255.

COTY, W.A. & McCONKEY Jr., C.L. (1982) A high-affinity calcium stimulated ATPase activity in the hen oviduct shell gland. Arch.Biochem.Biophys. 219: 444-453.

DHARMAMBA, M. & MAETZ, J. (1972) Effects of hypophysectomy and prolactin on the sodium of *Tilapia mossambica* in fresh water. Gen.Comp.Endocrinol. 19: 175-183.

DONEEN, B.A (1981) Effects of adaptation to sea water, 170% sea water, and to fresh water on activities and subcellular distribution of branchial Na^+-K^+-ATPase, low- and high-affinity Ca^{2+}-ATPase and ouabain insensitive ATPase in *Gillichthys mirabilis*. J.Comp.Physiol. 145: 51-61.

FENWICK, J.C. (1982) Pituitary control of calcium regulation. In: Comparative Endocrinology of calcium regulation (Oguro, C. and Pang, P.K.T. eds). Jap.Sci. Soc.Press, Tokyo: 13-19.

FLIK, G., WENDELAAR BONGA, S.E. & FENWICK, J.C. (1984c) Ca^{2+}-dependent phosphatase and Ca^{2+}-ATPase activities in plasma membranes of eel gill epithelium - III. Stimulation of branchial high-affinity Ca^{2+}-ATPase during prolactin induced hypercalcemia. Comp.Biochem.Physiol. In Press.

GHIJSEN, W.E.J.M. & VAN OS, C.H. (1979) Ca-stimulated ATPase in brush border and basolateral membranes of rat duodenum with high-affinity sites for Ca ions. Nature 279: 802-808.

GHIJSEN, W.E.J.M., DE JONG, M.D. & VAN OS, C.H. (1980) Dissociation between Ca^{2+}-ATPase and alkaline phosphatase activities in plasma membranes of rat duodenum. Biochim.Biophys.Acta 559: 538-551.

HO, S.M. & CHAN, D.K.O. (1980) Branchial ATPases and ionic transport in the eel *Anguilla japonica*. II. Ca^{2+}-ATPase. Comp.Biochem.Physiol. 67B: 639-645.

HOOTMAN, S.R. & PHILPOTT, C.W. (1978) Rapid isolation of chloride cells from pinfish gill. Anat.Rec. 190(3): 687-702.

HOOTMAN, S.R. & PHILPOTT, C.W. (1979) Ultracytochemical localization of Na^+/K^+-activated ATPase in chloride cells from the gills of a euryhaline teleost. Anat.Rec. 193: 99-130.

KARNAKY, K.J. Jr. (1980) Ion-secreting epithelia: chloride cells in the head region of *Fundulus heteroclitus*. Am.J.Physiol. 238: R185-R198.

KLAVEN, N.B., PERSHADSINGH, H.A., HENIUS, G.V., LARIS, P.C., LONG Jr., J.W. & McDONALD, J.M. (1983) A high-affinity, calmodulin-sensitive (Ca^{2+} + Mg^{2+})-ATPase and associated calcium-transport pump in the Ehrlich ascites tumor cell plasma membrane. Arch.Biochm.Biophys. 226(2): 618-628.

MA, S.W.T., SHAMI, Y., MESSER, H.H. & COPP, D.H. (1974) Properties of Ca^{2+}-ATPase from the gill of rainbow trout *(Salmo gairdneri)*. Biochim.Biophys.Acta 345: 243-251.

MA, S.W.Y. & COPP, D.H. (1981) Prolactin and calcium metabolism in teleosts. In: Hormonal control of Calcium metabolism. (Cohn, D.V., Talmage, R.V. and Matthews, J.L. eds) Excerpta Medica, Amsterdam: 425.

McCONNELL, D. (1973) Apatite. Its crystal chemistry, mineralogy, utilization and geologic and biologic occurrences. Springer Verlag Wien, New York.

MILET, C., HILLYARD, C.J., MARTELLY, E., GIRGIS, S., MacINTYRE, I. & LOPEZ, E. (1980) Similitudes structurales entre l'hormone hypocalcémiante des corpuscules de Stannius (PCS) de l'anguilla *(Anguilla anguilla* L.) et l'hormone parathyroidienne mammalienne. C.R. Acad.Sci.P. 291: 977-980.

OGASAWARA, T. & HIRANO, T. (1984) Effects of prolactin and environmental calcium on osmotic water permeability of the gills in the eel, *Anguilla japonica*. Gen. Comp.Endocrinol. 53: 315-325.

OLIVEREAU, M., AIMAR, C. & OLIVEREAU, J.M. (1980) PAS-positive cells of the pars intermedia are calcium-sensitive in the goldfish maintained in a hyposmotic milieu. Cell Tissue Res. 212: 29-38.

OLIVEREAU, M., OLIVEREAU, J.M. & AIMAR, C. (1981) Specific effects of calcium ions on the calcium-sensitive cells of the pars intermedia in the goldfish. Cell Tissue Res. 214: 23-31.

PANG, P.K.T., SCHREIBMAN, M.P. & GRIFFITH, R.W. (1973) Pituitary regulation of serum calcium levels in the killifish, *Fundulus heteroclitus* L. Gen.Comp. Endocrinol. 21: 536-542.

PANG, P.K.T., SCHREIBMAN, M.P., BALBONTIN, F. & PANG, R.K. (1978) Prolactin and pituitary control of calcium regulation in the killifish, *Fundulus heteroclitus*. Gen.Comp.Endocrinol. 36: 306-316.

PANG, P.K.T. & YEE, J.A. (1980) Evolution of hypercalcemic control. In: Hormones, Adaptation and Evolution (Ishii, S. *et al.*, eds). Japan Sci.Soc.Press Tokyo: 103-111.

PANG, P.K.T. (1981) Hypercalcemic effects of ovine prolactin on intact killifish, *Fundulus heteroclitus*, subjected to different environmental calcium challenges. Gen.Comp.Endocrinol. 43: 252-255.

PARFITT, A.M. (1979) Parathyroid hormone and the parathyroid glands. Metab.Bone Dis.Rel.Res. 1: 279-293.

PERSHADSING, H.A., McDONALS, M.L., LANDT, M., BRY, C.G., LACY, P.E., McDONALD, J.M. (1980) Ca^{2+}-activated ATPase and ATP-dependent calmodulin stimulated Ca^{2+}-transport in islet cell plasma membrane. Nature 288: 492-495.

RODGERS, D.W. (1984) Effects of ambient pH and calcium concentration on growth and calcium dynamics of brook trout, *Salvelinus fontinalis*. Can.J.Fish Aqua Sc. (submitted).

SASAYAMA, Y. & OGURO, C. (1982) Replacement therapy with pituitary homogenates or ovine prolactin on serum calcium, sodium and magnesium concentrations in bullfrog tadpoles. Gen.Comp.Endocrinol. 46: 75-80.

SHEPARD, K.L. (1981) The activity and characteristics of the Ca^{2+}-ATPase of fish gills in relation to environmental calcium concentrations. J.exp.Biol. 90: 115-121.

VAN OS, C.H. & GHIJSEN, W.E.J.M. (1981) High-affinity Ca-ATPase in basolateral plasma membranes of rat duodenum and kidney cortex. In: Calcium and phosphate transport across membranes (Bronner, F., Peterlik, M. eds). Academic Press, New York, 159-162.

WEISS, R.E. & WATABE, N. (1979) Studies on the biology of fish bone. -III. Ultrastructure of osteogenesis and resorption in osteocytic (cellular) and anosteocytic (cellular) bones. Calcif.Tissue Int. 28: 43-56.

WENDELAAR BONGA, S.E., VEENHUIS, M. (1974) The effect of prolactin on the number of membrane-associated particles in kidney cells of the euryhaline teleost *Gasterosteus aculeatus* during transfer from sea water to fresh water: a freeze-etch study. J.Cell Sci. 16: 687-701.

WENDELAAR BONGA, S.E. & VAN DER MEIJ, J.C.A. (1980) The effect of ambient calcium on prolactin cell activity and plasma electrolytes in *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. 40: 391-401.

WENDELAAR BONGA, S.E. & VAN DER MEIJ, J.C.A. (1981) Effect of ambient osmolarity and calcium on prolactin cell activity and osmotic water permeability of the gills in the teleost *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. 43: 432-442.

WENDELAAR BONGA, S.E. & FLIK, G. (1982) Prolactin and calcium metabolism in a teleost fish, *Sarotherodon mossambicus*. In: Comparative endocrinology of calcium regulation (Oguro, C., Pang, P.K.T. eds). Japan Scientific Societies Press, Tokyo, 21-26.

WENDELAAR BONGA, S.E. & LAMMERS, P.I. (1982) Effects of calcitonin on ultrastructure and mineral content of bone and scales of the cichlid teleost *Sarotherodon mossambicus*. Gen.Comp.Endocrinol. 48: 60-70.

WENDELAAR BONGA, S.E., LAMMERS, P.I. & VAN DER MEIJ, J.C.A. (1983a) Effects of 1,25- and 24,25-dihydroxyvitamin D_3 on bone formation in the cichlid teleost *Sarotherodon mossambicus*. Cell Tissue Res. 228: 117-126.

Het onderzoek dat beschreven staat in dit proefschrift heeft betrekking op de calcium-huishouding van vissen, in het bijzonder van vissen die in zoetwater leven. De twee soorten die gebruikt werden, de amerikaanse paling *(Anguilla rostrata* LeSueur) en afrikaanse natalbaars of tilapia *(Oreochromis mossambicus)*, zijn vertegenwoordigers van de beenvissen. Deze vissen bezitten een skelet dat gekenmerkt wordt door verkalkt bot. Onder natuurlijke omstandigheden groeien de meeste vissen voortdurend door. Ze moeten daarom calcium in hun botten opslaan. Calcium is echter niet alleen belangrijk voor de groei van het skelet, maar ook voor tal van andere fysiologische processen als spiersamentrekking, pulsgeleiding in zenuwen, het activeren van enzymen, bloedstolling en de productie van eieren. Bepalend voor het verloop van deze processen is het gehalte aan calcium ionen (Ca^{2+}) in het bloedplasma, dat nauwkeurig op een peil van ongeveer 1.5 mmol Ca^{2+} per liter plasma wordt gereguleerd. Deze nauwkeurige regeling van het calcium gehalte wordt aangeduid met calcium homeostase. Omdat over het algemeen de concentraties van ionen in de lichaamsvloeistoffen van de vis hoger zijn dan die van het hem omringende water, staat het dier voortdurend bloot aan het gevaar ionen, waaronder Ca^{2+}, te verliezen en treedt via osmose water het lichaam binnen. Het teveel aan water wordt dan weer verwijderd door veel, sterk verdunde, urine te produceren. Het verlies aan ionen wordt gecompenseerd door ionen op te nemen.

Een zoetwater vis beschikt over twee organen die hem in staat stellen Ca^{2+} op te nemen, namelijk de darm en de kieuwen. Uit onderzoek in de zestiger jaren is uit dieet-experimenten, waarbij vissen calcium-vrij voer kregen toegediend, gebleken dat een zoetwater vis het overgrote deel van het benodigde calcium als Ca^{2+} via zijn kieuwen uit het water opneemt. Opmerkelijk hierbij is dat de Ca^{2+}-concentratie in het water aanzienlijk kan varieren zonder dat dit veel effect heeft op het calcium gehalte van het bloed. Dit geeft aan dat het Ca^{2+}-opname systeem in de kieuwen onder een regulerende invloed van hormonen staat. Bij zeer lage calcium concentraties in het water treden evenwel verstoringen op die zich het beste laten beschrijven met de term "lek" worden. Dit verschijnsel belicht een speciaal voor vissen essentiele rol van calcium in de fysiologie van het dier, namelijk de instandhouding van de doorlaatbaarheid voor water en ionen van de cellen van de huid en de kieuwen. In dit opzicht is niet alleen de calcium concentratie in de lichaamsvloeistoffen van belang maar ook die in het water en wel speciaal voor die cellen die in direct contact staan met het water. Bij heel lage calcium concentraties in het water kan het Ca^{2+}-verlies van de

celmembranen zo groot worden dat de controle over water- en ionenbewegingen door de membranen verloren gaat: het dier wordt "lek". De regulatie van de doorlaatbaarheid van de kieuwen en de huid is niet alleen afhankelijk van de calcium concentratie in het water en in het bloedplasma, maar daarbij speelt ook het hormoon prolactine een rol. In een vis als tilapia is de productiviteit van de prolactine-cellen (in het hersenaanhangsel) omgekeerd evenredig met de calcium concentratie van het water. De rol van het prolactine in de calcium huishouding van de vis strekt zich nog verder uit, want het hormoon stimuleert ook de opname van Ca^{2+} uit het water.

Het onderzoek gerapporteerd in dit proefschrift is toegespitst op de vraag hoe de zoetwater vis via zijn kieuwen Ca^{2+} opneemt uit het water, welke orde grootte deze opname processen hebben en hoe het hormoon prolactine betrokken is bij deze opname van Ca^{2+} uit het water.

Het eerste deel van het proefschrift beschrijft biochemisch onderzoek naar de aard van Ca^{2+}-afhankelijke enzymen die betrokken zijn bij de opname van Ca^{2+} uit het water. Het Ca^{2+} dat uit het water via de kieuwen naar het bloed getransporteerd wordt passeert daarbij de zogenaamde chloride-cellen van het kieuwepitheel. Deze cellen zijn gespecialiseerd voor ionen transport. In deze cellen kon een Ca^{2+}-transporterend enzym worden aangetoond (hoofdstuk III), echter pas nadat een methode was ontwikkeld om plasma membranen van het kieuwepitheel te isoleren en bovendien onderscheid te maken tussen het echte Ca^{2+}-transporterende enzym en onspecifieke fosfatasen (hoofdstuk II). Het vierde hoofdstuk beschrijft dat toediening van prolactine aan palingen leidt tot een verhoging van het plasma calcium gehalte van deze dieren en deze verhoging gaat gepaard met een stimulatie van het transport enzym en laat zien hoe prolactine het Ca^{2+}-transport in de kieuwen kan stimuleren. In het vijfde hoofdstuk zijn een aantal resultaten samengevat met betrekking tot Ca^{2+}-transport mechanismen in de kieuwen van tilapia. Een vergelijkbaar Ca^{2+}-transport enzym als in de kieuwen van de paling werd aangetoond, maar bovendien dat de activiteit van dit enzym resulteert in accumulatie van Ca^{2+} in blaasjes van plasma membranen. Bij deze blaasjes was de cytoplasmatische kant van de celmembraan naar buiten gekeerd. De berekende orde grootte van dit plasma membraan-gebonden proces maakt een betrokkenheid ervan bij de opname van calcium uit het water zeer waarschijnlijk.

Het tweede deel van dit proefschrift betreft de calcium uitwisselings-processen tussen de vis en het water, en het belang ervan voor de calcium-homeostase en de groei. Ook de rol die het bot speelt in de calcium huishouding van de vis

wordt belicht. Met behulp van radioactieve calcium-isotopen en een "whole-body counter" die deze isotopen kan detecteren, werd de calcium uitwisseling bestudeerd in vrij-rondzwemmende vissen (hoofdstuk VI). Het blijkt dat de opname van Ca^{2+} uit het water het netto resultaat is van een naar binnen gerichte stroom (influx) en een naar buiten gerichte stroom (efflux) van Ca^{2+} via het kieuwweefsel. De influx is afhankelijk van actief transport van Ca^{2+} en opmerkelijk is dat deze influx van Ca^{2+} in grootte overeenkomst met de op grond biochemische gegevens berekende Ca^{2+}-transport capaciteit van de kieuwen. De efflux van Ca^{2+} wordt voornamelijk bepaald door de conditie van het kieuwweefsel, waarvoor op hun beurt het Ca^{2+}-gehalte van het water en het hormoon prolactine bepalend zijn. In normaal zoetwater blijkt dat in tilapia de influx van Ca^{2+} zoveel groter is dan de efflux dat de netto opname van Ca^{2+} uit het water voldoende is voor de groei. Deze conclusie is gebaseerd op analyses van het calcium gehalte in vissen van verschillende leeftijden en lichaamsgrootte. In hoofdstuk VII worden de gevolgen beschreven van verlaging van het calcium gehalte van het water voor de opname van Ca^{2+} via de kieuwen. Wanneer de Ca^{2+}-concentratie van het water wordt teruggebracht tot éénvierde van de oorspronkelijke concentratie, verliezen de vissen aanvankelijk calcium. Na een aantal weken blijken de dieren hun calcium opname systeem te hebben aangepast: de efflux neemt weliswaar toe, maar de influx neemt nog veel meer toe, wat resulteert in een verhoogde opname. In hoofdstuk VIII worden de effecten beschreven die het hormoon prolactine heeft op de opname van Ca^{2+} uit het water. Er kon worden aangetoond dat dit hormoon het calcium gehalte in het bloed verhoogt door de influx te stimuleren en de efflux te reduceren. De waarneming dat prolactine de opname van Ca^{2+} verhoogt ondersteunt de conclusie getrokken in hoofdstuk IV, dat de door prolactine gestimuleerde Ca^{2+}-ATPase activiteit direct betrokken is bij de opname van Ca^{2+} uit het water. De reductie van de Ca^{2+}-efflux toont aan dat het hormoon niet alleen de doorlaatbaarheid van kieuwen en huid voor water en eenwaardige ionen reguleert (zoals bekend was) maar ook die voor Ca^{2+}. De conclusie die uit alle verkregen resultaten getrokken kan worden is dat prolactine het belangrijkste hormoon is voor de calcium homeostase van zoetwater vissen, en wel omdat het de opname van Ca^{2+} uit het water controleert en daarmee garandeert dat het calcium gehalte van het bloed op het gewenste peil blijft.

In de algemene discussie (Hoofdstuk IX) wordt de regulatie van het calcium gehalte in het bloed bij vissen vergeleken met die van landdieren. Bij vissen kan het calcium gehalte continu worden geregeld, via stimulatie door prolactine van de calcium-opname uit het water. Bij landdieren komt calcium uitsluitend binnen met het voedsel. De calcium-opname is veelal een periodiek proces waarvan

de omvang niet nauwkeurig gereguleerd kan worden. Daardoor is het skelet van landdieren als calcium reservoir van veel groter belang voor de regulatie van het calcium gehalte in het bloed dan bij vissen. Het op peil brengen van het calcium gehalte van het bloed is met name afhankelijk van een hormoon uit de bijschildklieren (het parathormoon), dat calcium vrij maakt uit het bot. Bijschildklieren komen bij vissen niet voor. De conclusie uit deze gegevens is dat in de loop van de evolutie van de gewervelde dieren, tijdens de overgang van het leven in het water naar het leven op het land, het skelet in veel opzichten de rol van het water als calcium-reservoir heeft overgenomen. Hierdoor is waarschijnlijk de betekenis van prolactine voor de regulatie van de calcium-opname uit het milieu sterk verminderd. Bij landdieren heeft prolactine een belangrijke regulerende funktie gekregen bij de voortplanting.

Voor hun medewerking bij het tot stand komen van dit proefschrift wil ik graag bedanken:

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CURRICULUM VITAE

Gert Flik werd geboren op 6 februari 1954 te Zwolle. In 1973 behaalde hij het diploma gymnasium-β aan het Gymnasium Celeanum in zijn geboorteplaats.
In september van datzelfde jaar begon hij met de biologie-studie aan de Rijksuniversiteit Groningen, waarvoor het kandidaatsexamen (B1) in juni 1977 werd afgelegd. In december 1979 werd de studie afgesloten met het doctoraalexamen (cum laude): hoofdrichtingen Endocrinologie (mevrouw Prof. Dr. B. Baggerman, Dr. A.J.H. de Ruiter) en Gedragsfysiologie (Dr. A.B. Steffens); bijvakken Gedragsendocrinologie (Dr. T. Schuurman) en Immunologie (Dr. A.A. van den Broek).

In januari 1980 werd begonnen met het hiervoor beschreven onderzoek, uitgevoerd in het kader van de Universitaire Onderzoekpool (project WN 3/79) op de afdeling Dierfysiologie (Prof. Dr. A.P. van Overbeeke en Prof. Dr. S.E. Wendelaar Bonga) van de Katholieke Universiteit te Nijmegen. In december 1980 slaagde hij voor het examen "Deskundigheid Stralingshygiene voor een C-laboratorium". Gedurende de periode half mei tot half augustus 1982 werd een werkbezoek gebracht aan het laboratorium van Prof. Dr. J.C. Fenwick, Department of Biology, University of Ottawa, Ottawa, Canada. In september en oktober 1983 werd een werkbezoek gebracht aan het laboratorium van Dr. N. Mayer-Gostan, Laboratoire Jean Maetz, Villefranche-sur-Mer, Frankrijk.

Naast het doen van onderzoek werd een bijdrage geleverd aan het prekandidaats- en doctoraalonderwijs voor studenten in de biologie. Momenteel is hij verbonden als wetenschappelijk medewerker aan de afdeling Dierfysiologie van de Katholieke Universiteit te Nijmegen.

STELLINGEN

1. Voor het aantonen van ATP-hydrolytische activiteit van een transport Ca^{2+}-ATPase is de aanwezigheid van een Ca^{2+}-buffer en Mg^{2+} in het incubatiemedium vereist.
Dit proefschrift.

2. De localisatie van transport Ca^{2+}-ATPase activiteit op ultrastructureel niveau met behulp van "Wachstein-Meisel procedures" lijkt voorshands niet mogelijk.
Contra: Ando, T., Fujimoto, K., Mayahara, H., Miyajima, H. & Ogawa, K. (1981) Acta Histochem. Cytochem. 14(6): 705-726.

3. De conclusie van Berg, gebaseerd op zijn studies van het calcium- en strontium-metabolisme bij vissen, dat "Ca^{2+} in opposition to Na^{+} does not play any role in osmoregulation", is inmiddels achterhaald.
Berg, A. (1968) Me. Ist. Ital. Idrobiol. 23: 161-196.

4. Bij het gebruik van strontiumisotopen als calcium-tracer in biologische systemen dient men te bedenken dat de affiniteit van calcium-bindende eiwitten, zoals calmoduline, voor ionen van deze twee metalen verschillend is.

5. Gezien de eigenschappen van het kieuwepitheel van zoetwater vissen als "tight epithelium", moet een Ca^{2+}-influx van water naar bloed via de kieuwen beschouwd worden als een transcellulair proces. Een dergelijk proces wordt niet bepaald door de Ca^{2+}-gradient tussen water en bloed, maar veeleer door de beide Ca^{2+}-gradienten die over de apicale en de basolaterale plasma membranen van de cellen van dit epitheel staan.
Contra: Höbe, H., Laurent, P. & McMahon, B.R. (1984) J.exp. Biol. In press.

6. Het vóórkomen van calmoduline in slijm van vissen wijst erop dat dit eiwit niet uitsluitend als intracellulaire activator optreedt.
Flik, G., Van Rijs, J.H. & Wendelaar Bonga, S.E. (1984) Eur. J. Biochem. 138: 651-654.

7. De conclusie van Grau *et al.* dat de osmotische waarde van het water in belangrijke mate de prolactinecel activiteit van tilapia bepaald, is onvoldoende gefundeerd.
Grau, E.G., Nishioka, R.S. & Bern, H.A. (1981) Gen. Comp. Endocrinol. 45: 406-408.

8. De hoogte van noradrenaline spiegels in de laterale hypothalamus van de rat is bepalend voor de maaltijd-gebonden, neuronaal gestuurde afgifte van insuline door de B-cel van de endocriene pancreas.

Steffens, A.B., Flik, G., Kuipers, F., Lotter, E.C. & Luiten, P.G.M. (1984) Brain Research 310 (2): 351-362.

9. De histologische argumenten die door Lopez *et al.* worden aangevoerd in een poging om homologie van de bijschildklieren van hogere gewervelden met de lichaampjes van Stannius der vissen aan te tonen, zijn niet overtuigend.

In: Endocrine control of bone and calcium metabolism. D.V. Cohn et al., eds. Amsterdam, Elsevier Science Publishers B.V., 1984.

10. De schadelijke gevolgen van verzuring van het water voor de fysiologische functies van vissen zijn eerder te herleiden tot verstoring van de osmoregulatie dan tot een verstoorde ademhaling.

11. Het toxisch effect van cadmium op Ca^{2+}-afhankelijke processen in de cel zou in belangrijke mate kunnen worden veroorzaakt door het feit dat calmoduline hogere affiniteit voor Cd^{2+} dan voor Ca^{2+} vertoont, terwijl calmoduline-Cd^{2+} complexen niet als activator kunnen optreden.

Lock, R.A.C., Pärt, P., Van de Winkel, J.A.J. & Flik, G. (1984) The Biochemical Journal (Submitted).

12. Voer voor visfysiologen: niet alleen in het restaurant maar ook in het laboratorium geldt dat een vis van één kilogram niet altijd gelijk is aan één kilogram vis.

o.a. Dit Proefschrift.

Nijmegen, 27 september 1984

G. Flik